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# THE JOURNAL OF EXPERIMENTAL MEDICINE

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SIMON FLEXNER, M.D.

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1

# A CONSIDERATION OF THE RELATIVE TOXICITY OF URANIUM NITRATE FOR ANIMALS OF DIFFERENT AGES. I.\*

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PLATES 1 AND 2.

(Received for publication, January 31, 1917.)

The various ways in which the age of an organism expresses itself has received little consideration in the interpretation of many reactions that occur naturally and that are induced experimentally. The toxic effect of a substance experimentally introduced into an organism is usually interpreted either morphologically, by certain cell changes, or by some alteration in the functional capacity of an organ or a certain group of organs.

In a preliminary note<sup>1</sup> the observation was recorded that animals of different ages showed a variation in their response to the toxic effect of uranium nitrate when the poison was given subcutaneously in a constant quantity per kilo of body weight. The younger animals not only withstood the toxic effect of uranium for a longer period without developing an albuminuria and a glycosuria, but when these animals finally became both albuminuric and glycosuric, the quantitative output of these substances was much less in the younger than in the older animals.

In a more recent paper<sup>2</sup> the observation referred to has been confirmed in a second series of animals. A further observation has been made, that, judging by the time of appearance and the amount of acetone which occurred in the urine,

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\* Aided by a grant from The Rockefeller Institute for Medical Research.

<sup>1</sup> MacNider, W. deB., On the difference in the response of animals of different ages to a constant quantity of uranium nitrate, *Proc. Soc. Exp. Biol. and Med.*, 1913-14, xi, 159.

<sup>2</sup> MacNider, The inhibition of the toxicity of uranium nitrate by sodium carbonate, and the protection of the kidney acutely nephropathic from uranium from the toxic action of an anesthetic by sodium carbonate, *J. Exp. Med.*, 1916, xxiii, 171.

the older animals gave evidence of developing an acid intoxication much earlier than did the younger ones. In this paper it was also shown that a solution of sodium carbonate given intravenously would protect the kidney of an animal against the toxic effect of uranium, and that it was also possible to protect the kidney of a young animal acutely nephropathic from uranium against the toxic effect of an anesthetic by the use of a solution of sodium carbonate. The ability to furnish such a protection decreased as the age of the animal increased.

It is important to ascertain in a more detailed manner the way in which animals of different ages express their variation to the toxic effect of uranium. The present investigation is therefore primarily concerned with a study of acute uranium intoxications in animals of different ages as indicated by a disturbance in the metabolism of the animals, the severity of which shows a parallel with the age of the animal. During the course of such intoxications the animals become nephropathic. In these animals the kidney has been selected as an organ in which to study the functional and morphological variations which develop during the intoxication. The severity of these changes will be studied in relation to the age of the animal.

#### EXPERIMENTAL.

Dogs were employed in the experiments. The animals varied in age from pups of 8 months to dogs 8 years of age. The younger animals were raised in the laboratory kennels, while the older animals were obtained from people in the surrounding country who had raised the dogs and could vouch for their age within a few months.

The animals were placed in metabolism cages and given a liberal amount of bread with which was cooked a small amount of meat. The animals received 500 cc. of water daily by stomach tube. After a period of 3 days, which was allowed for normal observations, and during which time animals with a naturally acquired nephropathy or with glucose or acetone bodies in the urine could be excluded, the animals were given subcutaneously on 2 successive days 5 mg. of uranium nitrate per kilo of body weight. In all the animals the uranium intoxication was allowed to persist for 48 hours. At the end of this period the animals were either killed and autopsied, or the nephropathic animals were anesthetized and employed for cer-

tain functional studies which will be reported in Part II of this investigation.

The relative toxicity of uranium for these animals has been investigated by a study of the hydrogen ion content and alkali reserve of the blood, the tension of alveolar air carbon dioxide, and by the time of appearance and quantitative output of acetone and diacetic acid in the urine. The functional capacity of the kidney was ascertained by a study, at different periods during the intoxication, of the time of appearance and total output of phenolsulfonephthalein in the urine and by a study of the urea content of the blood.

The hydrogen ion determinations have been made by the indicator method recently devised by Levy, Rowntree, and Marriott.<sup>3</sup> The alkali reserve of the blood and the determinations of alveolar air carbon dioxide have been made by the methods of Marriott.<sup>4, 5</sup> The quantitative determinations of acetone and diacetic acid in the urine have been made by Folin's<sup>6</sup> method as modified by Hart.<sup>7</sup> The output of diacetic acid is expressed in terms of acetone. The blood urea determinations have been made by the method of Marshall,<sup>8</sup> following the modification suggested by Van Slyke and Cullen.<sup>9</sup> The phenolsulfonephthalein test for kidney function was conducted according to the method outlined by Rowntree and Geraghty.<sup>10</sup>

<sup>3</sup> Levy, R. L., Rowntree, L. G., and Marriott, W. McK., A simplified method for determining variations in the hydrogen ion content of the blood, *Arch. Int. Med.*, 1915, xvi, 389.

<sup>4</sup> Marriott, W. McK., A method for the determination of the alkali reserve of the blood plasma, *Arch. Int. Med.*, 1916, xvii, 840.

<sup>5</sup> Marriott, The determination of alveolar carbon dioxide tension by a simple method, *J. Am. Med. Assn.*, 1916, lxvi, 1594.

<sup>6</sup> Folin, O., On the separate determination of acetone and diacetic acid in diabetic urines, *J. Biol. Chem.*, 1907, iii, 177.

<sup>7</sup> Hart, T. S., On the quantitative determination of acetone in the urine, *J. Biol. Chem.*, 1908, iv, 477.

<sup>8</sup> Marshall, E. K., Jr., A rapid clinical method for the estimation of urea in urine, *J. Biol. Chem.*, 1913, xiv, 283.

<sup>9</sup> Van Slyke, D. D., and Cullen, G. E., A permanent preparation of urease, and its use in the determination of urea, *J. Biol. Chem.*, 1914, xix, 211.

<sup>10</sup> Rowntree, L. G., and Geraghty, J. T., An experimental and clinical study of the functional activity of the kidneys by means of phenolsulphonephthalein, *J. Pharm. and Exp. Therap.*, 1909-10, i, 579.

TABLE I.  
*Normal Animals.*

No. of experiment.	Age.	Weight.	Water in 24 hrs.	Urine in 24 hrs.	Sulfonephthalein.		Blood urea.	P <sub>H</sub> .	R. P <sub>H</sub> .	Carbon dioxide tension.	Albumin, glucose, acetone, diacetic acid.
					Time of appearance.	Output in 2 hrs.					
	yrs.	kg.	cc.	cc.	min.	per cent	per cent			mm.	
1	8 mos.	15.9	500	769	5	68	0.012	7.45	8.0	40	0
2	8 "	10.5	500	509	8	71	0.012	7.4	8.1	40	0
3	1	15.81	500	660			0.015	7.45	8.1	37	0
4	1	19.0	500	960	5	67	0.015	7.4	8.1	43	0
5	2	14.68	500	630			0.015	7.45	8.05	43	0
6	3+	17.01	500	670	5	67	0.015	7.45	8.0	44	0
7	3+	9.8	500	762	4½	67	0.015	7.4	8.0	38	0
8	4+	13.15	500	670			0.015	7.45	8.0	39	0
9	5+	13.3	500	465	4	66	0.012	7.45	8.05	40	0
10	5+	18.2	500	520			0.020	7.45	8.0	40	0
11	8	10.6	500	640	5	73	0.016	7.45	8.0	40	0
12	8+	8.53	500	430			0.015	7.45	8.0	39	0

TABLE II.  
*Nephropathic Animals after 24 Hours.*

No. of experiment.	Age.	Uranium nitrate per kilo.	Urine, 1st 24 hrs.	Sulfonephthalein.		Blood urea.	Acetone per 100 cc.	Diacetic acid per 100 cc.	P <sub>H</sub> .	R. P <sub>H</sub> .	Carbon dioxide tension.
				Time of appearance.	Output in 2 hrs.						
	yrs.	mg.	cc.	min.	per cent	per cent	mg.	mg.			mm.
1	8 mos.	5	483	4	64	0.012	0	0	7.45	8.0	38
2	8 "	5	704	5	66	0.012	0	0	7.4	8.1	35
3	1	5	995			0.015	0	0	7.45	8.1	35
4	1	5	745	5	64	0.015	0	0	7.45	8.1	43
5	2	5	459			0.015	0	0	7.45	8.05	35
6	3+	5	1,150	7	54	0.016	0	0	7.35	8.0	40
7	3+	5	435	3½	51	0.015	0	0	7.4	8.0	35
8	4+	5	360			0.015	0	0	7.3	7.95	34
9	5+	5	1,060	8	31	0.015	0	0	7.4	7.95	36
10	5+	5	1,300			0.015	0	0	7.4	7.9	36
11	8	5	645	10	17	0.016	2.6109	3.4812	7.4	7.9	37
12	8+	5	520			0.015	2.6592	2.7559	7.25	7.9	34



TABLE III.

*Nephropathic Animals after 48 Hours.*

No. of experiment.	Age.	Uranium nitrate per kilo.	Urine, 2nd 24 hrs.	Sulfonephthalein.		Blood urca.	Acetone per 100 cc.	Diabetic acid per 100 cc.	P <sub>H</sub> .	R. P <sub>H</sub> .	Carbon dioxide tension.
				Time of appearance.	Output in 2 hrs.						
	yrs.	mg.	cc.	min.	per cent	per cent	mg.	mg.			mm.
1	8 mos.	5	769	5	20	0.012	2.9493	2.2756	7.4	8.0	37
2	8 "	5	509	7	18	0.012	1.3054	4.0614	7.4	8.0	35
3	1	5	660			0.020	3.8680	2.3208	7.4	8.0	35
4	1	5	960	11	21	0.020	2.3691	4.3015	7.35	8.0	39
5	2	5	630			0.020	2.8043	4.1099	7.35	7.9	30
6	3+	5	670	10	24	0.020	1.4010	4.2064	7.35	7.9	35
7	3+	5	762	21	4	0.042	2.3045	4.1092	7.3	7.85	31
8	4+	5	595			0.026	3.7862	4.6416	7.3	7.85	32
9	5+	5	465	20	8	0.022	3.3845	9.6700	7.4	7.9	35
10	5+	5	520			0.022	0.3703	2.2241	7.3	7.85	34
11	8	5	640	20	4	0.030	4.9800	2.2241	7.25	7.85	34
12	8+	5	430			0.030	2.6592	7.3975	7.2	7.8	30

Tables I, II, and III give the normal findings in these animals of different ages for 1 normal day and for the 2 subsequent days of the uranium intoxication. The tables show the results obtained in twelve of the animals which have been selected according to their age as representative of the total number of animals employed in the experiments.

#### *Observations on Normal Animals of Different Ages.*

All the animals during the period of 3 days allowed for normal observations were freely diuretic. The total output of urine for the last day of observation varied in the respective animals from a minimum of 430 cc. to a maximum output of 960 cc. The urine was free from albumin, acetone bodies, and glucose, and did not contain casts.

Table I shows that in conducting the sulfonephthalein test for kidney function, the appearance of the dye in the urine varied slightly in the different animals. The earliest appearance was 4 minutes following the injection, while in one animal the appearance of the

dye was delayed for 8 minutes. The total output of the dye in a 2 hour period varied between a minimum of 66 per cent to a maximum output of 73 per cent. These variations have apparently no connection with the age of the animal. The highest output of the dye in this series of animals occurred in an animal 8 years old (Experiment 11, Table I).

The percentage of blood urea has been very constant for animals of all ages. In the normal dog the urea content per 100 cc. of blood has varied between 0.012 to 0.020 per cent.

The hydrogen ion determinations have been made from the oxalated whole blood. In such determinations the hydrogen ion concentration expresses both the volatile and non-volatile acid content of the blood. Recently Marriott<sup>4</sup> has shown that frequently such readings are misleading, and that a more accurate conception of the changes in the hydrogen ion concentration of the blood may be obtained by removing the carbon dioxide from the dialysate, in this way obtaining a reading which represents any change in hydrogen ion concentration which may be due to non-volatile acids. Marriott refers to such a reading as the reserve alkali content of the blood ( $R. P_H$ ).

In the normal animals of all ages the hydrogen ion concentration of the whole blood ( $P_H$ ) has varied between 7.3 to 7.45, while the alkali reserve of the blood ( $R. P_H$ ) has varied between 8.0 to 8.1.

The determinations of the tension of alveolar air carbon dioxide have shown a variation within the limits of normality, 37 to 44 mm. The Marriott method for such determinations was employed in seventy-one dogs and has given remarkably constant results.

From the observations which have been made on the normal animals we may conclude that the animals of different ages show no appreciable difference in their ability to eliminate sulfonephthalein, that the blood urea content in these animals is very constant, and that the animals even though varying much in age show naturally no tendency towards an acid intoxication.

*Observations on Animals of Different Ages Intoxicated by Uranium Nitrate.*

In the following study of the relative toxicity of uranium, it first became necessary to ascertain whether the weight of the animal, and therefore the total amount of the poison introduced, had any effect in determining the toxicity of the substance. By referring to Tables II and III it will be seen that this factor apparently does not influence the toxic response on the part of the animals. For instance the pup of Experiment 1, with a weight of 15.9 kilos, showed a delayed and slight toxic effect from uranium, while the animal of Experiment 12, 8 years old, and with a weight of only 8.53 kilos, showed clearly the toxic action of uranium during the first 24 hours following the initial injection, and by the end of the second 24 hour period was severely intoxicated. This type of observation has remained constant in all the animals employed in this study.

Following the first injection of uranium all the animals remained freely diuretic. In several of the animals (Experiments 6, 9, and 10) the output of urine was greatly increased.

A study of the sulfonephthalein output by the animals of different ages shows the following variations: There is only a slight change in the time of the appearance of the dye in the urine as compared with the normal animals. The delay in the time of appearance has been most marked in the older animals, while in the youngest animals the time of appearance has either been increased over the normal or remained unchanged.

In the animals of all ages the total elimination of sulfonephthalein in a 2 hour period is reduced. The reduction is slight in the young animals and marked in the old animals. In Experiment 1, a pup 8 months old, the total output of the dye was only reduced from the normal of 68 to 64 per cent. In Experiment 11, an animal 8 years old, a reduction occurred from the normal of 73 to 17 per cent.

Following the second injection of uranium there is a continuation of the relatively greater toxic effect of this poison for the kidneys of the older animals. The youngest animals which have been recorded in Tables II and III were 8 months old and from the same litter. These animals at the end of the 48 hour period of intoxication by

uranium had a sulfonephthalein output of 20 and 18 per cent respectively. The two oldest animals in which sulfonephthalein determinations were made show an output of the dye of 8 and 4 per cent.

The toxic effect of uranium for the kidney as shown by the reduction in the output of sulfonephthalein increases with the age of the animals.

The determinations of blood urea in the pathological as compared with the normal animals enable observations to be made concerning the degree of urea retention in the animals of different ages and also permit a study of the relation between the output of sulfonephthalein by the kidney and the amount of urea retained.

Following the first injection of uranium the percentage of blood urea remained practically constant in all the animals. There was no evidence of a retention of urea even though the output of sulfonephthalein had been greatly reduced. In Experiment 7, Table II, the sulfonephthalein output was reduced to 51 per cent while the percentage of blood urea remained constant. In Experiment 11, the sulfonephthalein output was reduced following the first injection of uranium from 73 to 17 per cent. The percentage of blood urea was uninfluenced by this degree of kidney injury and remained at the normal reading of 0.016 per cent.

Following the second injection of uranium a retention of blood urea was found to occur in all the animals over 8 months old. The animals which show the greatest reduction in the output of sulfonephthalein also show the highest retention of blood urea. The tables of experiments furthermore show that the decrease in the functional capacity of the kidney as indicated by a retention of blood urea increases with the age of the animal.

From these observations it would appear that as compared with the sulfonephthalein test for kidney function, the retention of blood urea is a much later manifestation of kidney inefficiency. When, however, the kidney shows serious impairment of function as indicated by the sulfonephthalein test, there occurs a retention of blood urea which shows a parallel with this test for renal function.

The degree to which the kidney may be impaired and yet show no evidence of a retention of blood urea is illustrated by the first two experiments of Table III. The output of sulfonephthalein by these

two young animals was 20 and 18 per cent, respectively, and yet the percentage of blood urica showed no variation from the normal.

In a previous paper<sup>2</sup> on uranium intoxications in animals of different age it was shown that the time of the appearance of acetone bodies in the urine and the relative amount of these bodies increased with the age of the animal. This observation was interpreted as indicating the development of an acid intoxication, and furthermore to furnish ground for the belief that such an intoxication was more readily induced by uranium in an old animal than in a young animal. This interpretation was strengthened by a series of experiments in which it was found possible to protect an animal against the toxic effect of uranium by the use of an alkaline solution intravenously. The degree of protection conferred by such injections was largely dependent upon the age of the animal. Young animals were more readily protected than were old animals.

Howland and Marriott<sup>11</sup> have recently called attention to the fact that the presence of acetone bodies in the urine is in itself insufficient evidence of a tissue acidosis. It is furthermore well known that in conditions of tissue acidosis the output of these bodies in the urine may be reduced as a result of a decrease in the functional capacity of the kidney. It has therefore seemed advisable in this series of animals to ascertain whether there was any evidence of an acid intoxication other than that shown by the appearance of acetone bodies in the urine. With this object in view determinations of the hydrogen ion content of the blood, the alkali reserve of the blood, and the carbon dioxide tension of alveolar air have been made to determine first, if by these methods any evidence can be obtained of a tissue acidosis, second, if the degree of acid intoxication shows any parallel with the quantitative output of acetone bodies in the urine, and, finally, if the degree of intoxication as indicated by these different methods shows any variation with the age of the animal.

Reference to Table II shows that following the first injection of uranium none of the animals under 8 years of age had a urine which contained acetone or diacetic acid. However, the two oldest ani-

<sup>11</sup> Howland, J., and Marriott, W. McK., A discussion of acidosis. With special reference to that occurring in diseases of children, *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 63.

mals of the series, Experiments 11 and 12, one 8 years old and the other 8 years and a few months old, showed both acetone and diacetic acid in the urine at this early period of the uranium intoxication. By referring to Table II it will also be observed that the appearance of acetone and diacetic acid in the urine of the two oldest animals coincides with the development of other indications of an acid intoxication. These animals also show an increase in the hydrogen ion concentration of the blood, a reduction in the alkali reserve of the blood, and a decrease in the tension of alveolar air carbon dioxide.

A study of the animals of Experiments 8, 9, and 10, which have varied in ages between  $4\frac{1}{2}$  and 5 years and 2 months, shows that an acid intoxication may exist without the appearance of acetone bodies in the urine. All these animals had a urine which was free from both acetone and diacetic acid and yet all three of the animals gave other evidence of a beginning acid intoxication. It would therefore seem that the appearance of acetone and diacetic acid in the urine may indicate a beginning acid intoxication from uranium. On the other hand, the absence of these bodies from the urine does not exclude a tissue acidosis, for when other tests are employed such a state may be found to exist.

During the second 24 hours of the uranium intoxication the animals of all ages showed the presence of acetone and diacetic acid in the urine. In so far as the development of an acid intoxication can be determined by the presence of these substances in the urine, at this stage of the intoxication the tendency of the animals to develop an acidosis has extended so as to include not only the old animals but the animals of all ages.

The quantitative output of acetone and diacetic acid shows no constant increase with the increasing age of the animal. In general the combined acetone and diacetic acid output is greater in an old animal than in a young animal, but throughout the series of experiments numerous instances have been observed in which there is no true correlation between the age of the animal and the acetone and diacetic acid content of the urine. For example, in Experiment 2, Table III, in a pup 8 months old, the urine, following the second injection of uranium, contained 1.3054 mg. of acetone and 4.0614

mg. of diacetic acid in terms of acetone per 100 cc. of urine. In Experiment 10, in an animal 5 years and 2 months old, the output of acetone was only 0.3703 mg. per 100 cc. of urine, and the output of diacetic acid which was 2.2241 mg. per 100 cc. was but slightly over half the quantity found in the urine of the young animal.

A study of the relation between the quantitative output of acetone bodies in the urine and the changes in blood and alveolar air shows that when these bodies appear in the urine changes also occur in the blood and alveolar air indicative of a beginning acid intoxication.

The experiments also show that at this stage of the intoxication there is no correlation between the total output of acetone bodies and the other indications of the development of a tissue acidosis. For instance, in the animal of Experiment 10, at the end of the uranium intoxication the hydrogen ion content of the blood had been increased to 7.3, the alkali reserve of the blood was reduced to 7.85, and the tension of carbon dioxide in alveolar air gave a reading of 34 mm. The urine, however, showed a remarkably low output of both acetone and diacetic acid, acetone 0.3703 mg. and diacetic acid 2.2241 mg. per 100 cc. of urine.

From this review of the output of acetone and diacetic acid by animals of different ages intoxicated by uranium, the following conclusions may be drawn. The oldest animals show the toxic effect from uranium by the appearance of these bodies in the urine 24 hours before they appear in the urine of the younger animals. When animals of any age show the presence of these substances in the urine, they also show other evidence of a beginning acid intoxication.

The acid intoxication which develops from uranium cannot be solely ascribed to the formation of acetone bodies. These substances may fail to appear in the urine when there is other evidence of a beginning acid intoxication, and when as shown by the sulfonephthalein test the kidney has not become functionally inactive to such a degree as to cause a retention of these bodies. We must therefore conclude that the acid intoxication which develops from uranium is certainly dependent in part upon the formation or retention of acids other than those of the acetone series. Finally, it has been shown that the quantitative output of acetone and diacetic acid bears no constant relation to the age of the animal, and also that there is no

definite correlation between the quantitative output of acetone bodies and the degree of acid intoxication which may be demonstrated by other tests which have been employed for this purpose.

The variation in the toxicity of uranium for animals of different ages is more clearly expressed in changes in the acid-base equilibrium of the blood than by any other response which has been induced during the course of the intoxication.

Reference to Table II shows that following the first injection of uranium the animals under 4 years of age maintain a hydrogen ion content of the blood which varies only slightly from the normal. None of these animals have shown any change in the alkali reserve of the blood. In contrast with this indication of stability on the part of the animals under 4 years of age, all the animals over 4 years old have not only shown an increase in the hydrogen ion content of the blood but the alkali reserve of the blood has constantly shown a depletion. In these older animals in which the alkali reserve has been reduced, the tension of carbon dioxide in alveolar air has varied between 37 to 34 mm. In the younger animals in which the alkali reserve has remained unaffected the tension of carbon dioxide has varied between 43 and 35 mm.

The relative degree of acid intoxication induced in the animals of different ages is even more clearly shown following the second injection of uranium. By this stage of the intoxication, all the animals over 1 year of age not only show an increase in the hydrogen ion content of the blood, but the alkali reserve of the blood has been severely drawn upon. The younger animals, those under 1 year of age, have either shown no change in the hydrogen ion content of the blood, or in the few experiments in which these readings have varied, the hydrogen ion content has not been increased above 7.4, a point which may be considered within the limit of normal variations. In these younger animals the alkali reserve of the blood has either remained unaffected, or it has not been reduced below 8.0.

A study of Tables II and III not only indicates the differences in the hydrogen ion content and alkali reserve of the blood, which, as has been pointed out, exist between the animals of two age limits, those under 1 year of age and those over 1 year of age, but it also serves to demonstrate that as the animal increases in age there is a pro-



gressive increase in the degree of acidosis. For instance, at the end of the 2nd day of the uranium intoxication the animal of Experiment 6, 3 years and 2 months old, had a hydrogen ion concentration of 7.35 and an alkali reserve of 7.9, while the animal of Experiment 12, 8 years old, had a hydrogen ion content of 7.2 and an alkali reserve of 7.8.

The determinations of carbon dioxide tension in alveolar air of the animals of different ages at the end of the 2nd day of the uranium intoxication show that the older animals that have developed a reduction in the alkali reserve of the blood have an alveolar air carbon dioxide tension which varies between 35 to 30 mm. The younger animals in which no change has taken place in the alkali reserve, or in which the alkali reserve has not been reduced below 8.0, show a tension of carbon dioxide which varies between 39 to 35 mm.

From the observations the conclusion appears clear that one of the constant manifestations of the toxic effect of uranium is the development of an acid intoxication, and that the severity of this intoxication is associated with the age of the animal. The older animals develop a severer intoxication than do the younger animals.

*The Pathology of the Kidney in Animals of Different Ages Intoxicated by Uranium.*

At the end of the 2nd day of the uranium intoxication, twelve of the animals which have been employed in the experiments were killed and kidney tissue was at once fixed in 10 per cent formalin, Zenker's fluid, and in mercuric chloride-acetic acid to be used in the histological study. The animals from which tissue was obtained varied in age between 8 months, and 7 years and 4 months.

In the foregoing discussion of the relative toxicity of uranium in animals of different ages it has been shown that the toxicity of uranium increases with the age of the animal, and furthermore that the degree of toxicity of this substance is associated with the severity of the acid intoxication which develops in these animals. The following histological study has been undertaken with the object of ascertaining whether there is any pathological difference in the kidneys of animals which have shown very mild grades as contrasted

with severe grades of acid intoxication. The functional tests that have been employed have shown a correlation between the degree of acid intoxication and the ability of the kidney to eliminate sulfone-phthalein and urea.

In the histological study it has not been found possible to differentiate between kidneys which were obtained from animals near the same age. In kidneys which were obtained from animals that varied 3 years or more in age there has been found such a difference in the pathological response of the organs as to permit a classification of the kidneys into two groups. The first group is represented by kidneys of animals not over 1 year of age and the second group by kidneys of animals that were over 3 years of age.

The kidneys of the younger animals show no evidence of damage to the vascular tissue. The glomerular capillaries are usually well filled with blood. No exudate has been observed in the subcapsular space or between the tubules. The connective tissue of the kidney has not been edematous. The endothelial nuclei of the glomerular vessels and of the cells lining the capsule of the glomerulus stain normally and show no evidence of degeneration or of proliferation.

The epithelium of the tubules, and especially of the convoluted tubules, shows a definite shrinkage. The lumen of the tubules is prominent and usually free from albuminous material. The nuclei of these cells are large in proportion to the surrounding cytoplasm and stain intensely (Fig. 1).

No stainable fat has been observed in the convoluted tubules. In frozen sections of the kidney stained for fat by Herxheimer's modification of the Scharlach R stain, there has been found in the ascending and descending limb of Henle's loop a very small amount of fat which appears as dust-like particles.

The pathology of the kidney of the older group of animals shows a similarity with the younger group in that no demonstrable changes have taken place in the vascular element of the kidney. The epithelium of the tubules shows the following changes which serve to separate the kidneys of the animals of different ages into the two groups.

The cells of the convoluted tubules show an increase in volume which is variable. In some of the animals the swelling has been marked, while in other animals this change has taken place to a

much less extent. Associated with the swelling of the cells the cytoplasm becomes distinctly granular and occasionally shows vacuolation. The free border of the cells appears ragged. The nuclei are decreased in size and stain less intensely (Fig. 2).

In such cells, unless the animal is very old, no stainable fat has been demonstrated. However, in the loops of Henle the amount of stainable fat has greatly increased over that which has been observed in the younger group of animals. The fat appears as large droplets which frequently coalesce. The difference in the amount of stainable fat in the kidneys of these two groups of animals of different ages is the most constant and striking histological variation.

When these differences in the pathology of the kidney are compared with the variations in the degree of acid intoxication and the alterations in the functional capacity of the kidney that are shown by the two groups of animals, the following differences are found to exist.

The kidneys of the younger group of animals in which the epithelium is histologically well preserved except for the appearance of a slight amount of fat in the tubules of Henle show only slight evidence of a beginning tissue acidosis and the functional capacity of the kidney is much less impaired than is the case with the second group of older animals.

The kidneys of the older animals which give histological evidence of a beginning degeneration of the convoluted tubules and which have shown a marked accumulation of fat in the tubules of Henle, show a much severer grade of acid intoxication than the younger animals, and also show that the functional capacity of the kidney has been severely impaired.

In the older group of animals there is an association between the degree of epithelial injury and the amount of fat in the kidney, with the severity of the acid intoxication, and the extent to which the functional capacity of the kidney has been affected.

As a result of this observation the question naturally arises: Does the kidney injury develop primarily, and the tissue acidosis result from a retention of acid bodies which the kidney is unable to remove, or is the kidney injury secondary to, and dependent upon the acid intoxication, resulting from the administration of uranium?

This question has been in a measure answered in the communication<sup>2</sup> previously referred to. If young animals that are being intoxicated by uranium are given a solution of sodium carbonate intravenously they fail to develop an acid intoxication and the renal epithelium either shows no evidence of degeneration, or the degenerative changes are slight when compared with the epithelial damage which occurs in animals unprotected by the use of an alkali.

#### CONCLUSIONS.

1. The toxic effect of uranium when given in a constant quantity per kilo of body weight is variable. This variation has been constantly associated with differences in the age of the animals. Uranium is more toxic for an old animal than for a young animal. The establishment of this fact, namely, that the age of an animal may modify the toxicity of a substance, should be taken into account in establishing by animal experiment the degree of activity of substances which are to be used for therapeutic purposes.

2. The toxic effect of uranium nitrate is constantly associated with its ability to induce a tissue acidosis. A severer grade of acidosis is induced in an old animal from uranium than is induced in a young animal.

It would appear that in the response of dogs of different ages to uranium the animals represent a reaction system to this substance which shows an increasing susceptibility as the animal advances from youth to senility.

Insufficient experimental data are as yet available to allow a discussion of the mechanism by which such an acid intoxication is produced.

3. The toxic effect of uranium is manifested locally by certain degenerative changes in the kidney. These changes are more marked in the kidney of an old animal than they are in the kidney of a young animal.

Associated with the severer kidney changes which are especially characterized by a beginning swelling of the renal epithelium and by an accumulation of stainable fat in these cells is the development of a severe grade of tissue acidosis.

4. The functional capacity of the kidney shows a parallel with the degree of acid intoxication and with the severity of the histological changes which have developed in the renal epithelium.

#### EXPLANATION OF PLATES.

##### PLATE 1.

FIG. 1. Camera lucida drawing, Leitz oc. 1, obj. 6. The figure is from the kidney of the young dog of Experiment 3. The glomerular vessels are well filled with blood. The epithelium of the convoluted tubules, *a*, shows a distinct shrinkage; the nuclei are large and stain intensely. The lumen of the tubules is prominent. At *b* are shown tubules in which the epithelium is beginning to show an early swelling. The animal gave little evidence of an acid intoxication.

##### PLATE 2.

FIG. 2. Camera lucida drawing, Leitz oc. 1, obj. 6. The figure is from the old animal of Experiment 11. The glomerulus shows no degenerative changes. The epithelium of the convoluted tubules, *a*, appears granular, stains less intensely than the epithelium of the younger animal of Fig. 1, and the nuclei are small and hypochromatic. The free borders of the cells are ragged. At *b* are shown tubules in which the cells are swollen. At *c* the epithelium shows vacuolation. The animal had developed a severe acid intoxication.



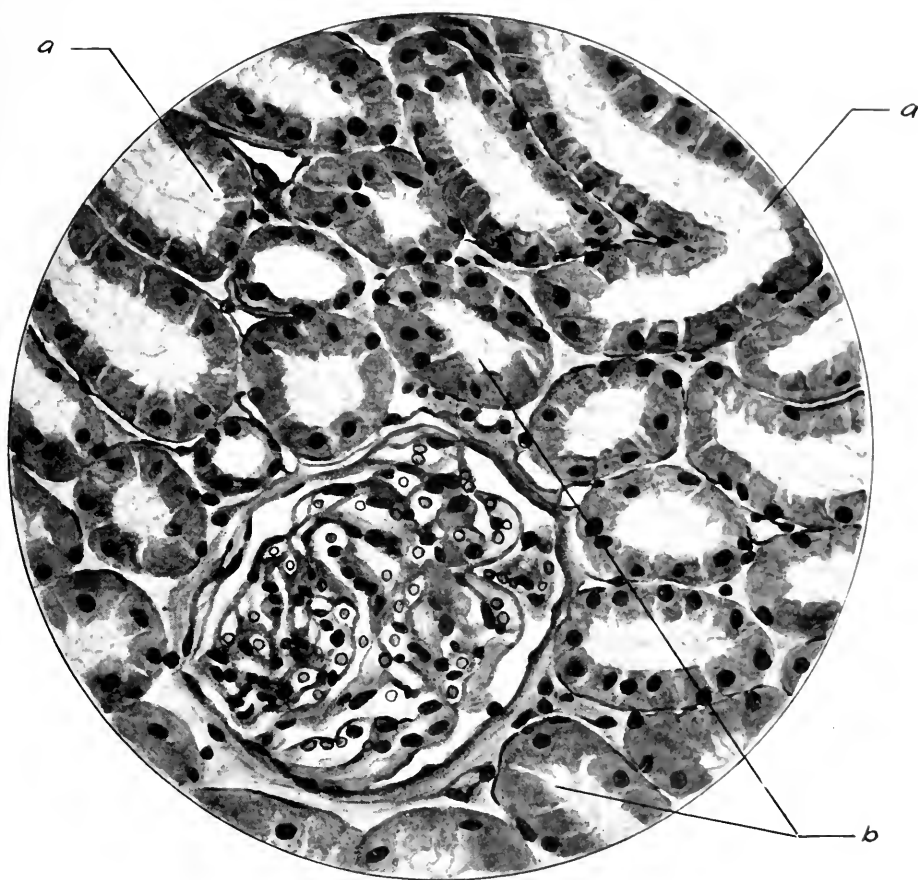


FIG. 1.

(MacNider: Toxicity of uranium. I.)





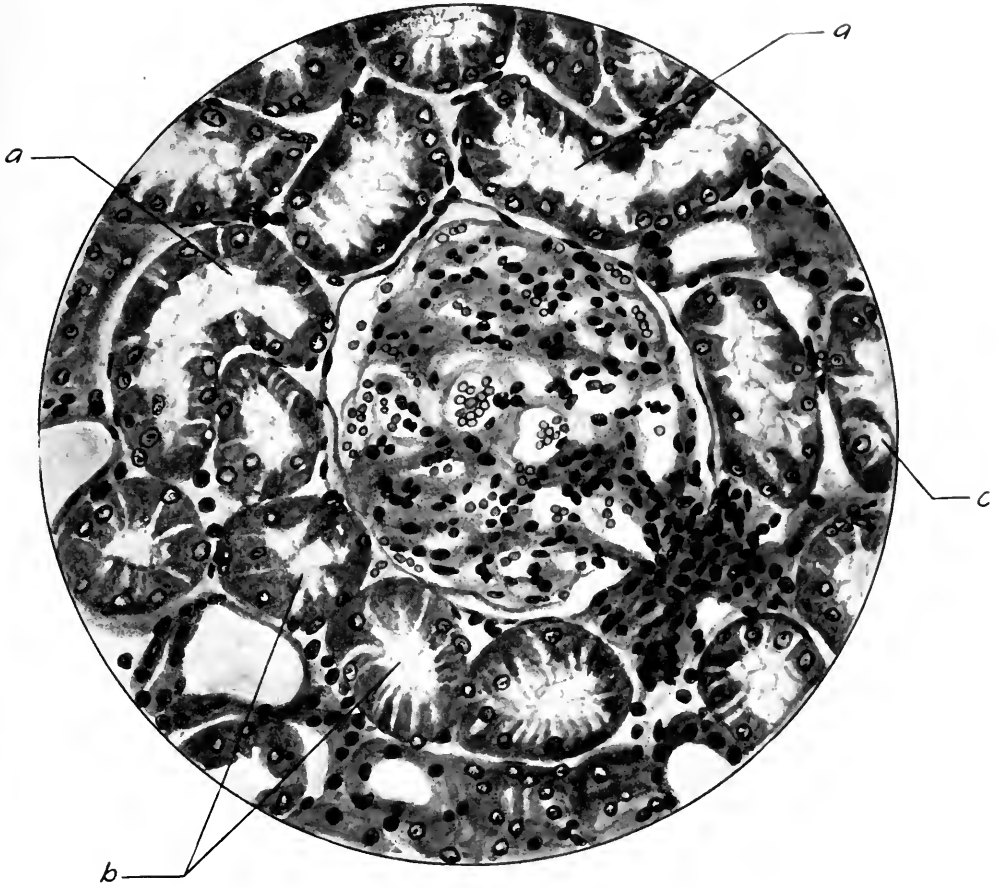


FIG. 2.

(MacNider: Toxicity of uranium. I.)



THE EFFICIENCY OF VARIOUS DIURETICS IN THE  
ACUTELY NEPHROPATHIC KIDNEY, PROTECTED  
AND UNPROTECTED BY SODIUM  
CARBONATE. II.\*

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PLATES 3 TO 6.

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In a recent paper<sup>1</sup> it was shown that intravenous injections of a solution of sodium carbonate inhibited the toxic action of uranium nitrate for the kidney. It was furthermore shown in this paper that the use of such an alkaline solution would protect the kidney of young animals acutely nephropathic from uranium against the toxic effect of an anesthetic. Animals protected in such a manner formed a much larger quantity of urine in a given time limit than did animals of the same age which had received the same amount of uranium nitrate per kilo but which received in place of a solution of carbonate a 0.9 per cent solution of sodium chloride. A histological study of the kidneys of these two groups of animals showed that the animals receiving the carbonate protection failed to develop severe degenerative changes in the renal epithelium, while those animals which were not protected by the carbonate had a renal epithelium which was acutely swollen and undergoing necrosis. In these experiments no information was obtained which would explain the cause of the swelling and necrosis of the epithelium or the relative efficiency of an alkaline solution to furnish a protection against an anesthetic as compared with the efficiency of an equal volume of a solution of sodium chloride.

In Part I of the present study I have been able to show by inducing uranium intoxications in animals of different ages that the severity of the uranium intoxication was dependent upon the degree of acid

\* Aided by a grant from The Rockefeller Institute for Medical Research.

<sup>1</sup> MacNider, W. deB., The inhibition of the toxicity of uranium nitrate by sodium carbonate, and the protection of the kidney acutely nephropathic from uranium from the toxic action of an anesthetic by sodium carbonate, *J. Exp. Med.*, 1916, xxiii, 171.

intoxication which developed in the different animals, and furthermore that the older animals developed a severer acid intoxication than did the younger animals.

The object of the present study is twofold: first, to determine the changes in the acid-base equilibrium of the blood induced by an anesthetic in normal animals, in animals acutely nephropathic from a constant quantity of uranium per kilo and protected by the intravenous use of an alkali, and in animals nephropathic from the same quantity of uranium but unprotected by an alkali; second, in such a series of animals to study the efficiency of various diuretics and the relative severity of the pathological changes which have taken place in the kidney.

#### EXPERIMENTAL.

Forty-six animals were used in this series of experiments. The animals varied in age from pups 8 months old to animals 11 years and 4 months old. Thirty of the animals that were employed in these experiments were used in Part I of this paper. The animals of the present series that are not included in these experiments were subjected to an experimental technique similar to that previously described. Ten of the animals were normal dogs which varied in age from 1 to 5 years. These animals were kept in metabolism cages for 3 days before they were used for experimental purposes, were fed on bread with a small amount of meat, and were given 500 cc. of water daily by stomach tube. The remaining thirty-six nephropathic animals were given the same diet and the same amount of water.

Tables I and II furnish an outline of the observations which have been made on two of the normal animals and thirteen of the acutely nephropathic animals. The animals have been selected according to their age in order to show the influence of this factor in determining the toxicity of an anesthetic and in determining the ability of an alkali to protect the nephropathic kidney against an anesthetic. In the experiments on nephropathic animals, two dogs were used in each experiment. One of the animals received the alkaline solution, while the other animal of the same age received an equal volume of sodium chloride solution and served as a control. On the day of the experiment, prior to the use of an

TABLE I.

No.	Experiment.	Age.	Uranium per kilo on 2 successive days.		Urine on day of ex- periment.	P <sub>H</sub> .	R. P <sub>H</sub> .	Carbon dioxide ten- sion.	Na <sub>2</sub> CO <sub>3</sub> , 3 per cent, or NaCl, 25 cc. per kilo.	Gréhan's anesthetic.	P <sub>H</sub> 1st half hr.		Carbon dioxide ten- sion 1st half hr.	Urine flow per min. 1st half hr.
			yrs.	mg.	cc.			mm.		per cent			mm.	drops
1	Normal.	1	0	690	7.45	8.1	39	0		60	7.45	8.1	39	2
2	"	4	0	635	7.45	8.1	43	0		60	7.45	8.1	42	2
3	Carbonate.	8 mos.	5	769	7.25	8.0	34	Na <sub>2</sub> CO <sub>3</sub> .		60	7.5	8.1	39	8
4	Control.	8 "	5	509	7.4	8.0	35	NaCl equimo- lecular with Na <sub>2</sub> CO <sub>3</sub> .		60	7.35	7.95	34	0
5	Carbonate.	11 "	5	723	7.35	8.0	35	Na <sub>2</sub> CO <sub>3</sub> .		60	7.45	8.2	40	1
6	Control.	11 "	5	765	7.45	8.0	36	NaCl equimo- lecular with Na <sub>2</sub> CO <sub>3</sub> .		60	7.4	7.9	32	0
7	Carbonate.	1	5	660	7.4	8.0	34	Na <sub>2</sub> CO <sub>3</sub> .		60	7.45	8.1	37	7
8	Control.	1	5	960	7.35	8.0	33	NaCl equimo- lecular with Na <sub>2</sub> CO <sub>3</sub> .		60	7.3	7.9	30	0
9	Carbonate.	1½	5	575	7.25	7.9	33	Na <sub>2</sub> CO <sub>3</sub> .		60	7.55	8.2	40	3
10	Control.	1½	5	415	7.35	7.95	33	NaCl equimo- lecular with Na <sub>2</sub> CO <sub>3</sub> .		60	7.3	7.9	30	0
11	Carbonate.	3+	5	362	7.3	7.85	31	Na <sub>2</sub> CO <sub>3</sub> .		60	7.5	8.05	40	0
12	Control.	3+	5	470	7.3	7.9	35	NaCl equimo- lecular with Na <sub>2</sub> CO <sub>3</sub> .		60	7.25	7.9	35	0
13	Carbonate.	5+	5	398	7.25	7.85	26	Na <sub>2</sub> CO <sub>2</sub> .		60	7.35	8.0	45	0
14	"	8	5	340	7.25	7.85	30	"		60	7.5	8.0	45	0
15	"	8	5	310	7.2	7.8	28	"		60	7.45	8.0	45	0

anesthetic, the hydrogen ion content of the blood, the alkali reserve of the blood, and the tension of alveolar air carbon dioxide were determined by the methods previously described. The nephropathic animals were then given intravenously either 25 cc. per kilo of a 3 per cent solution of sodium carbonate or the same amount of a solution of sodium chloride equimolecular with 3 per cent sodium carbonate. The normal animals were not given either of these solutions. In such animals it was possible to obtain information cover-

TABLE II.

No.	Experiment.	Diuretic.	Urine flow per min. 2nd half hr.	P <sub>H</sub> 2nd half hr.	R. P <sub>H</sub> 2nd half hr.	Urine flow per min. 2nd half hr.	Diuretic solution.	Urine flow per min. 3rd half hr.	P <sub>H</sub> 3rd half hr.	R. P <sub>H</sub> 3rd half hr.	Carbon dioxide ten- sion 3rd half hr.	Urine flow per min. 3rd half hr.
			drops			drops		drops			mm.	drops
1	Normal.	Theobromine, 1%	7	7.4	8.1	3	Urea 0.9%	64	7.45	8.1	38	7
2	"	Pituitrin, 0.5 cc.	10	7.3	8.1	5	NaCl 5%	10 cc.	7.2	8.0	39	5
3	Carbonate.	" 0.5 "	28	7.35	8.1	10	Urea 0.9%	38	7.3	8.0	35	20
4	Control.	" 0.5 "	0	7.35	7.9	0	" 0.9%	0	7.35	7.9	29	0
5	Carbonate.	Theobromine, 1%	4	7.35	8.1	1	NaCl 5%	22	7.35	8.05	37	6
6	Control.	" 1%	0	7.25	7.85	0	" 5%	0	7.25	7.85	21	0
7	Carbonate.	" 1%	12	7.5	8.0	5	" 5%	41	7.3	7.95	30	16
8	Control.	" 1%	0	7.2	7.8	0	" 5%	0	7.2	7.8	27	0
9	Carbonate.	" 1%	13	7.35	8.0	5	Urea 0.9%	44	7.3	8.0	35	21
10	Control.	" 1%	0	7.2	7.8	0	" 0.9%	0	7.1	7.75	23	0
11	Carbonate.	" 1%	0	7.3	7.9	0	NaCl 5%	0	7.25	7.8	29	0
12	Control.	" 1%	0	7.1	7.8	0	" 5%	0	7.05	7.75	24	0
13	Carbonate.	" 1%	0	7.3	7.85	0	" 5%	0	7.2	7.8	26	0
14	"	" 1%	0	7.3	7.9	0	" 5%	0	7.2	7.8	28	0
15	"	" 1%	0	7.3	7.9	0	" 5%	0	7.2	7.8	26	0

ing the natural susceptibility of the kidney to the anesthetic. All the animals were anesthetized by Gréhant's<sup>2</sup> anesthetic in 60 per cent strength. When the animals had become completely anesthetized the abdomen was opened and cannulas were tied into both ureters. The flow of urine was recorded in drops per minute. Half an hour after giving the anesthetic observations were made on the acid-base equilibrium of the blood and these observations were repeated every half hour during the experiment. The experiments were terminated at the end of 1½ hours. At each half hour period during the experiments the animals were given, intravenously, one of the following diuretic solutions, a record being made of the relative efficiency of these substances in the different groups of animals: theobromine, 1 per cent, 1 cc. per kilo; pituitrin (Parke, Davis and

<sup>2</sup> The animal is given 0.25 cc. per kilo of a 4 per cent solution of morphine. This is followed in half an hour by 10 cc. per kilo of the following mixture: chloroform, 50 cc.; alcohol and water, each 500 cc.

Company), 0.5 cc. regardless of weight; urea solution, 0.9 per cent, in 0.9 per cent sodium chloride, 10 cc. per kilo; sodium chloride, 5 per cent, 10 cc. per kilo.

*The Efficiency of Diuretics in the Normal Animal Anesthetized by Gréhant's Anesthetic.*

In Experiment\* 1 and 2 of Tables I and II an outline is given of the blood changes, the changes in carbon dioxide tension, and the efficiency of several types of diuretic substances in two of the ten normal animals which were anesthetized with Gréhant's anesthetic. The animals were respectively 1 and 4 years old. Both these animals were freely diuretic prior to the anesthetic. The hydrogen ion content of the blood, the alkali reserve, and the tension of carbon dioxide in alveolar air were normal. Half an hour after the animals were anesthetized no change had taken place in these readings except a reduction of the tension of carbon dioxide of 1 mm. The urine flow from each animal was two drops per minute. The animal of Experiment 1 was given 1 cc. per kilo of a 1 per cent solution of theobromine, while the animal of Experiment 2 received 0.5 cc. of pituitrin. The flow of urine was increased by theobromine to seven drops per minute and by pituitrin to ten drops per minute. At the end of the second half hour period no change had taken place in the alkali reserve of the blood. The combined volatile and non-volatile acid content of the blood as indicated by the hydrogen ion determinations had increased in the animal 1 year old from 7.45 to 7.4, while in the animal 4 years old this reading was 7.3, as compared with the normal of 7.45. At this stage of the anesthesia the animal of Experiment 1 was given 10 cc. per kilo of a 0.9 per cent solution of urea, and the animal of Experiment 2, 10 cc. per kilo of a 5 per cent solution of sodium chloride. The diuresis from both these substances was marked. The urea solution increased the flow of urine to sixty-four drops per minute and the sodium chloride solution to 10 cc. per minute. At the end of the experiments,  $1\frac{1}{2}$  hours after the animals were anesthetized, the flow of urine from both animals was in excess of the normal. In the young animal of Experiment 1 no change had taken place during the period of anesthesia in the hydrogen ion con-

tent or alkali reserve of the blood and practically no change in the tension of alveolar air carbon dioxide. The tension had been reduced 1 mm. In the animal of Experiment 2, 4 years old, the hydrogen ion content had increased from the normal of 7.45 to 7.2 and the alkali reserve showed a reduction from 8.1 to 8.0. The tension of carbon dioxide had decreased from the normal of 43 to 39 mm.

The experiments on the normal animals show that the normal kidney is relatively non-susceptible to the toxic effect of Gréhan's anesthetic. Animals anesthetized by this mixture, the active anesthetic ingredients of which are chloroform and alcohol, remain diuretic during a period of  $1\frac{1}{2}$  hours and respond to such diuretic substances as theobromine, pituitrin, and solutions of urea and sodium chloride. The experiments also indicate that in the older normal animals there is a tendency for the anesthetic to increase the hydrogen ion content of the blood and reduce the alkali reserve of the blood. The extent to which these changes have developed in the normal animals has not been indicated by any reduction in the response of the kidneys to various diuretics.

At the end of the experiments on normal animals and prior to the death of the animals the kidneys were removed and studied histologically. The findings were negative.

*The Efficiency of Diuretics in Nephropathic Animals Anesthetized by Gréhan's Anesthetic and Protected against the Anesthetic by a Sodium Carbonate Solution as Compared with Animals of the Same Age Which Were Unprotected.*

Thirteen of the thirty-six experiments on nephropathic animals are included in Tables I and II. Ten of the experiments were conducted in pairs, one animal receiving intravenously prior to the anesthetic 25 cc. per kilo of a 3 per cent solution of sodium carbonate, while the other animal which was used as a control was given an equivalent volume per kilo of a solution of sodium chloride equimolecular with the carbonate solution. All the animals had been intoxicated by uranium in the dose of 5 mg. per kilo which was given on the 2 days prior to the day of the experiment. During the last day of the uranium intoxication all the animals were diuretic. The output of urine varied between a minimum of 310 cc. to a maximum



of 960 cc. The urine was collected at 9 a.m. and the animals were anesthetized for the experiments in the afternoon of the same day. At this time the hydrogen ion and alkali reserve determinations were made. These determinations have therefore no association with the output of urine as recorded in Table I. It will, however, be noted that the older animals with a well marked acid intoxication show a decrease in the output of urine.

A study of the animals included in Table I, before they were anesthetized, confirms the observation made in Part I of this investigation. The older animals at the end of a 2 day period of intoxication by uranium show a severer grade of acid intoxication than do the younger animals. The animals over  $1\frac{1}{2}$  years of age show a more constant increase in the hydrogen ion content of the blood, and the alkali reserve of the blood has been drawn upon to a greater extent than has been the case with the animals under  $1\frac{1}{2}$  years of age. The carbon dioxide tension shows a greater reduction in the older animals than in the young animals.

Following these determinations the animals were given either the carbonate solution or the solution of sodium chloride, and at once anesthetized with Gréhan's anesthetic in 60 per cent strength. The observations which follow were made at half hour intervals during the experiments.

Reference to the tables of experiments shows that depending upon the relative susceptibility of the kidney to this anesthetic the animals may be divided into two groups. (1) The control animals. All these animals, even though they had first received 25 cc. per kilo of a solution of sodium chloride, became anuric by the end of the first half hour period of the experiment. This applies to animals of all ages. (2) The animals which received a solution of sodium carbonate. These animals may be subdivided into two groups. (a) The nephropathic animals not over  $1\frac{1}{2}$  years old which received the alkaline solution remained diuretic during the experiments, while (b) those animals over this age became anuric and remained anuric during the entire experiment. A study of the changes in the blood and alveolar air of these different groups of animals shows the following variations.

Within half an hour following the anesthetic the control animals

show an increase in the hydrogen ion content of the blood and a beginning reduction in the alkali reserve of the blood. The tension of carbon dioxide either remains constant or is decreased. In the control animals in this short period the anesthetic has increased the degree of acid intoxication which existed prior to the use of the anesthetic. All the animals that received the carbonate solution show at this period of the experiment a decrease in the hydrogen ion content of the blood, an increase in available alkali, and an increase tension of carbon dioxide. Those animals over  $1\frac{1}{2}$  years old which resisted the toxic effect of the anesthetic and remained diuretic show a more marked decrease in the hydrogen ion content of the blood and a larger amount of available alkali than do the animals over this age which became anuric from the anesthetic. For instance, in the diuretic animal of Experiment 9 the carbonate solution changed the hydrogen ion reading prior to the anesthetic from 7.25 to 7.55 and the alkali reserve from 7.9 to 8.2. In the anuric animal of Experiment 13 the hydrogen ion determination was changed by the carbonate from 7.25 to 7.35 and the alkali reserve from 7.85 to 8.0. The inability of the alkaline solution to protect the nephropathic kidney against the anesthetic is associated with its failure to decrease the acid intoxication of the anuric animals to the same extent to which the intoxication is modified in the diuretic group.

At this stage of the experiments, the end of the first half hour period, two of the animals were given pituitrin and the remaining eleven animals were given theobromine. The control animals remained uniformly anuric from these diuretic substances. The carbonate-protected animals under  $1\frac{1}{2}$  years of age which had not shown a severe acid intoxication prior to the anesthetic and which following the anesthetic and the intravenous injection of the alkaline solution gave no evidence of an acid intoxication were freely diuretic to both pituitrin and theobromine. The older animals, those over  $1\frac{1}{2}$  years of age, which prior to the anesthetic had shown a severe grade of acid intoxication and which following the anesthetic and the carbonate solution gave less evidence of protection against the acid intoxication have remained anuric and unresponsive to these diuretic substances. A solution of sodium carbonate is able to protect the kidney of a nephropathic animal against Gréchant's anesthetic up to

a certain age limit. After this age limit is reached the older animals are not afforded a protection by the carbonate but become anuric as do the control animals which have received a solution of sodium chloride.

A study of the tables of experiments from this first half hour period following the anesthetic, through the remaining periods to the termination of the experiments, shows the variations which occur in the acid-base equilibrium of the blood in the control animals, in the carbonate-protected animals which remain diuretic, and in those animals receiving the carbonate which become anuric; it also shows the relative efficiency of diuretics in these three groups of animals.

It will be observed that all the control animals remain anuric through the remaining hour of the respective experiments and that none of these animals show any diuretic effect from a 0.9 per cent solution of urea or a 5 per cent solution of sodium chloride. It will also be observed that in the control animals the degree of acid intoxication tends to increase progressively in severity as the age of the animal increases. The control animal of Experiment 4, 8 months old, at the end of the experiment had a hydrogen ion content of 7.35 and an alkali reserve of 7.9. The carbon dioxide tension was 29 mm. The control animal of Experiment 12, 3 years and 7 months old, had a hydrogen ion content of 7.05, an alkali reserve of 7.75, and a carbon dioxide tension of 24 mm. Nephropathic animals anesthetized by Gréhant's anesthetic and unprotected against the toxic action of the anesthetic by an alkali show the same type of response to the anesthetic as the animals of different ages have shown to uranium. The toxicity of the anesthetic increases with the age of the animal.

During the remainder of the experiments the nephropathic animals which received the sodium carbonate protection permit the same division into two groups. The animals not over  $1\frac{1}{2}$  years of age remain diuretic to the conclusion of the experiment, while those over this age remain anuric.

At the end of the second half hour period of the experiments all the animals which had received the carbonate protection were given either 10 cc. per kilo of a 0.9 per cent solution of urea or 10 cc. per kilo of a 5 per cent solution of sodium chloride. The functional response of the kidneys to these diuretics was clear-cut. The younger

group of animals which were previously diuretic to either theobromine or pituitrin and which at the present stage of the experiment showed a hydrogen ion concentration not over 7.35 and an alkali reserve not below 8.0 were freely diuretic to both 5 per cent sodium chloride and a 0.9 per cent solution of urea. The older animals, which earlier in the experiments had shown no diuretic effect from theobromine, at this later stage of the experiment also remained anuric to the solutions of urea and sodium chloride. In this latter group of anuric animals the injected carbonate solution had been rapidly used up so that the blood showed an alkali reserve of not over 7.8.

During the course of the anesthesia all the animals which have received the carbonate protection show an increase in the hydrogen ion content of the blood and a reduction in the alkali reserve. The degree to which these changes take place varies in the diuretic and anuric groups of animals. In the younger group of animals which remain diuretic the reduction in the alkali of the blood takes place gradually so that during an anesthesia lasting  $1\frac{1}{2}$  hours the alkali reserve of the blood has not shown a reading below 7.95 and the hydrogen ion content has not been over 7.35. The older group of animals in which no protection was afforded by the sodium carbonate solution and which remained anuric throughout the period of anesthesia have shown a rapid depletion of the blood of the injected alkali, so that by the termination of the experiment this group of animals has had a hydrogen ion content as high as 7.2, and an alkali reserve as low as 7.8.

The changes in carbon dioxide tension in alveolar air of the two groups of animals have shown in general a correlation with the change in the alkali reserve of the blood. In the group of young animals which show less reduction in the alkali reserve the tension of carbon dioxide has varied between 30 to 37 mm. In the older group of animals with a more marked reduction in the alkali reserve the tension of carbon dioxide has varied between 26 to 29 mm.

The foregoing brief review of the differences in the acid-base equilibrium of the blood in the diuretic and anuric groups of animals shows that the establishment of a state of anuria in an animal nephropathic from uranium and anesthetized by Gréhan's anesthetic is associated with the development of a tissue acidosis and with a

failure of the carbonate solution to maintain the reaction of the blood near the point of neutrality.

*The Pathology of the Kidney in Nephropathic Animals, Protected and Unprotected against the Toxic Effect of Gréhan's Anesthetic by Sodium Carbonate.*

At the termination of the experiments and before the death of the animals the kidneys were removed for histological study. Tissue was fixed in 10 per cent formalin and in mercuric chloride-acetic acid. Sections for routine histological study were stained with eosin and hematoxylin. Frozen sections were stained for fat by Herxheimer's modification of the Scharlach R stain.

Depending upon the severity of the pathological changes which have developed in the kidneys, these organs may be classified into three groups: the kidneys of animals which have received the carbonate protection and which have remained diuretic and responsive to diuretic substances; the kidneys of animals which also received the carbonate protection but which during the development of the anesthesia became anuric and remained unresponsive to diuretic substances; and finally, the group of control animals all of which following the anesthetic became anuric.

The kidneys of the animals which were successfully protected by the carbonate show no degenerative change in the glomerular vessels. These vessels are usually distended with blood and when compared with the glomerular vessels in the anuric group of animals the difference in the degree of distention of the loops is very evident. The epithelium of the convoluted tubules is shrunken. The nuclei of these cells are large and hyperchromatic. The lumen of the tubules is prominent. Occasionally tubules are observed in which the cells show an accumulation of granules, or more rarely vacuoles, and an early swelling. Stainable fat has not been found in the epithelium of the convoluted tubules, the glomerular vessels, or in the connective tissue of the kidney. The loops of Henle usually show a trace of fat (Figs. 1 and 2).

The kidneys of those animals which received the carbonate solution but which were not afforded any protection against the toxic effect

of the anesthetic show a pathological response which is striking when compared with the changes in the kidneys that have been protected by the alkaline solution. The vascular element of the kidney shows no evidence of degeneration. The capillary loops of the glomeruli are certainly not engorged with blood, and when compared with the glomerular vessels of the previous group of animals protected by the carbonate, the glomerular vessels of this anuric group contain less blood. The epithelium of the convoluted tubules is severely swollen. The cell cytoplasm has not become necrotic and the nuclei have not undergone fragmentation. In many of the tubules the lumen has become completely obliterated by the swollen cells (Fig. 3). The epithelium of the convoluted tubules has not contained any clearly defined stainable fat. The loops of Henle show a large amount of fat in the form of droplets, which frequently fuse together.

The kidneys of the control animals, which instead of receiving the alkaline solution were given a solution of sodium chloride equimolecular with the carbonate solution, show a pathological response resembling that of the anuric group of animals which failed to receive any protection from the carbonate solution. The pathological changes in the kidneys of this control group show more advanced evidence of degeneration. The glomerular vessels are not distended with blood. The cells of the convoluted tubules are severely swollen, granular, and frequently hydropic. The nuclei are hypochromatic and undergoing fragmentation. The cytoplasm of many of the cells has become necrotic (Fig. 4). In these cells stainable fat has frequently been demonstrated. The relative amount of fat in the loops of Henle is far greater in this control group of animals which were anuric than in the anuric group of animals that received the carbonate solution.

#### SUMMARY.

The outline which has been given of the relative toxicity of Gréhan's anesthetic in normal animals, in animals that were nephropathic from uranium and protected against the anesthetic by an alkaline solution, and in those animals which were unprotected by such a solution, furnishes the basis for the following summary.

The kidney of the normal dog is relatively non-susceptible to the

toxic action of Gréhan's anesthetic. The kidneys have failed to show any change in their histological structure during the period of anesthesia. These animals have remained diuretic during the period of anesthesia and have responded to diuretics such as theobromine, pituitrin, and solutions of urea and sodium chloride.

Normal animals anesthetized with Gréhan's mixture for  $1\frac{1}{2}$  hours usually show at the end of the experiment either no change, or only a slight variation from the normal, in the hydrogen ion content of the blood, the alkali reserve of the blood, and in the tension of carbon dioxide. In several normal dogs which were over 4 years of age, by the end of an anesthesia of such a duration the animals have shown a reduction in the alkali reserve of the blood and also a decrease from the normal in the carbon dioxide tension of alveolar air. From this observation it would appear that even in a normal animal Gréhan's anesthetic tends to induce an acid intoxication, and as was the case with normal animals which were being intoxicated by uranium, such an intoxication is more readily induced in an old animal than in a young one.

The nephropathic animals which have been anesthetized by Gréhan's anesthetic and in which an attempt has been made to protect these animals against the toxic effect of the anesthetic by the use of a solution of sodium carbonate fall into two clear-cut groups. Those animals of the series not over  $1\frac{1}{2}$  years old have shown at the end of the uranium intoxication and prior to the use of the anesthetic a less severe acid intoxication than have the animals of the series which were over  $1\frac{1}{2}$  years old. In this younger group of animals the intravenous injection of a 3 per cent solution of sodium carbonate immediately before the animals were anesthetized has succeeded in protecting these animals against the toxic action of the anesthetic. During the following  $1\frac{1}{2}$  hours of anesthetization these animals have not developed a severe grade of acid intoxication, and in several of the animals at the end of the experiment the alkali reserve of the blood was in excess of what it was at the end of the uranium intoxication and before an anesthetic was administered. Animals of this protected group have remained diuretic throughout the experiment and have shown an active diuresis from pituitrin, theobromine, and solutions of urea and sodium chloride. The kidneys of such animals

have shown histologically a normal vascular tissue, a convoluted tubule epithelium which gave the appearance of being hyperactive, and only occasionally were tubules encountered which showed signs of an early epithelial degeneration.

The nephropathic animals of the series in which a solution of sodium carbonate failed to afford any protection against Gréhan's anesthetic were animals over  $1\frac{1}{2}$  years old in which the uranium intoxication had resulted in a severer grade of acid intoxication than in the younger animals. When these older animals were given intravenously the carbonate solution and were anesthetized, it was found impossible to increase the alkali reserve of the blood to the same extent as was possible in the younger animals. Furthermore, the alkaline solution during the period of anesthesia is rapidly used up so that by the termination of these experiments the animals may have an alkali reserve of the blood which may be even lower than was the alkali reserve before the use of the carbonate. These animals have remained completely anuric throughout the experiments and have shown no diuretic effect from those diuretics which in the animals that were successfully protected by the carbonate induced free diuresis. The kidneys of these anuric animals show no degenerative changes in the glomerular vessels. The capillaries are not distended with blood as has been the case with the diuretic group. The epithelium of the convoluted tubules is acutely swollen. The swelling has frequently taken place to such an extent that the lumen of the tubules has become obliterated.

The nephropathic animals of the series which served as control animals and which were given a solution of sodium chloride equimolecular with the carbonate solution, following Gréhan's anesthetic became completely anuric. The sodium chloride solution furnished no protection against the anesthetic. The animals of all ages became anuric and unresponsive to the diuretic substances which have been used during this study. With the establishment of a state of anuria in these control animals the hydrogen ion content of the blood has increased, the alkali reserve of the blood has been rapidly depleted, and associated with this change the carbon dioxide tension has been reduced. The rapidity with which these changes develop



and the degree of acid intoxication which is induced is more marked in these animals than in any of the other series.

The kidneys of the control animals show the severest grade of degeneration of any of the nephropathic animals. The epithelium of the convoluted tubules is not only severely swollen but the cells frequently show necrosis. The loops of Henle contain more stainable fat than has been demonstrated in the kidneys of the carbonate animals.

#### CONCLUSIONS.

1. The toxicity of Gréhant's anesthetic for the nephropathic kidney is associated with the ability of the anesthetic to induce an acid intoxication. The severity of the acid intoxication increases with the age of the animal.

2. The efficiency of a solution of sodium carbonate to protect the kidney against the toxic effect of Gréhant's anesthetic depends upon its ability to prevent the development of an acid intoxication. The efficiency of a solution of sodium carbonate to furnish such a protection decreases as the age of the animal increases.

3. The inability of a solution of sodium carbonate to protect the kidney against Gréhant's anesthetic has been characterized by the development of an acute swelling of the renal epithelium which is later followed by necrosis. These animals develop an acute anuria and are unresponsive to diuretics.

4. The ability of a solution of sodium carbonate to protect the kidney against Gréhant's anesthetic has been associated with the histological preservation of the renal epithelium. These animals remain diuretic and responsive to various diuretic substances.

5. The inference should not be drawn from the observations which have been made that the epithelial damage is the sole cause for the anuria and the lack of response of some of the animals to diuretic substances. It is difficult to conceive that such gross changes in the volume of the renal epithelium could occur without seriously affecting the functional response of the vascular mechanism of the kidney.

## EXPLANATION OF PLATES.

## PLATE 3.

FIG. 1. Camera lucida drawing, Zeiss oc. 3, obj. 6. The figure is from the kidney of the carbonate animal of Experiment 3, Table I. The glomerular vessels show no degeneration and the capillary loops are well filled with blood. The epithelium of the convoluted tubules, *a*, is shrunken; the lumen of the tubules is prominent. The nuclei are large and hyperchromatic. At *b* the epithelium shows an early swelling. The animal was successfully protected against Gréhan's anesthetic by the carbonate solution, and was freely diuretic to both pituitrin and a 0.9 per cent solution of urea.

## PLATE 4.

FIG. 2. Camera lucida drawing, Zeiss oc. 3, obj. 6. The figure is from the kidney of the carbonate animal of Experiment 5, Table I. The pathology of the kidney is in general similar to that shown in Fig. 1. The glomerular vessels fill the subcapsular space and the capillary loops are well filled with blood. The epithelium of the convoluted tubules, *a*, is shrunken; the nuclei of the cells are large and hyperchromatic. At *b* these cells show a beginning swelling. The animal was successfully protected against the anesthetic by the alkaline solution; it had a marked diuresis from both theobromine and a 5 per cent solution of sodium chloride.

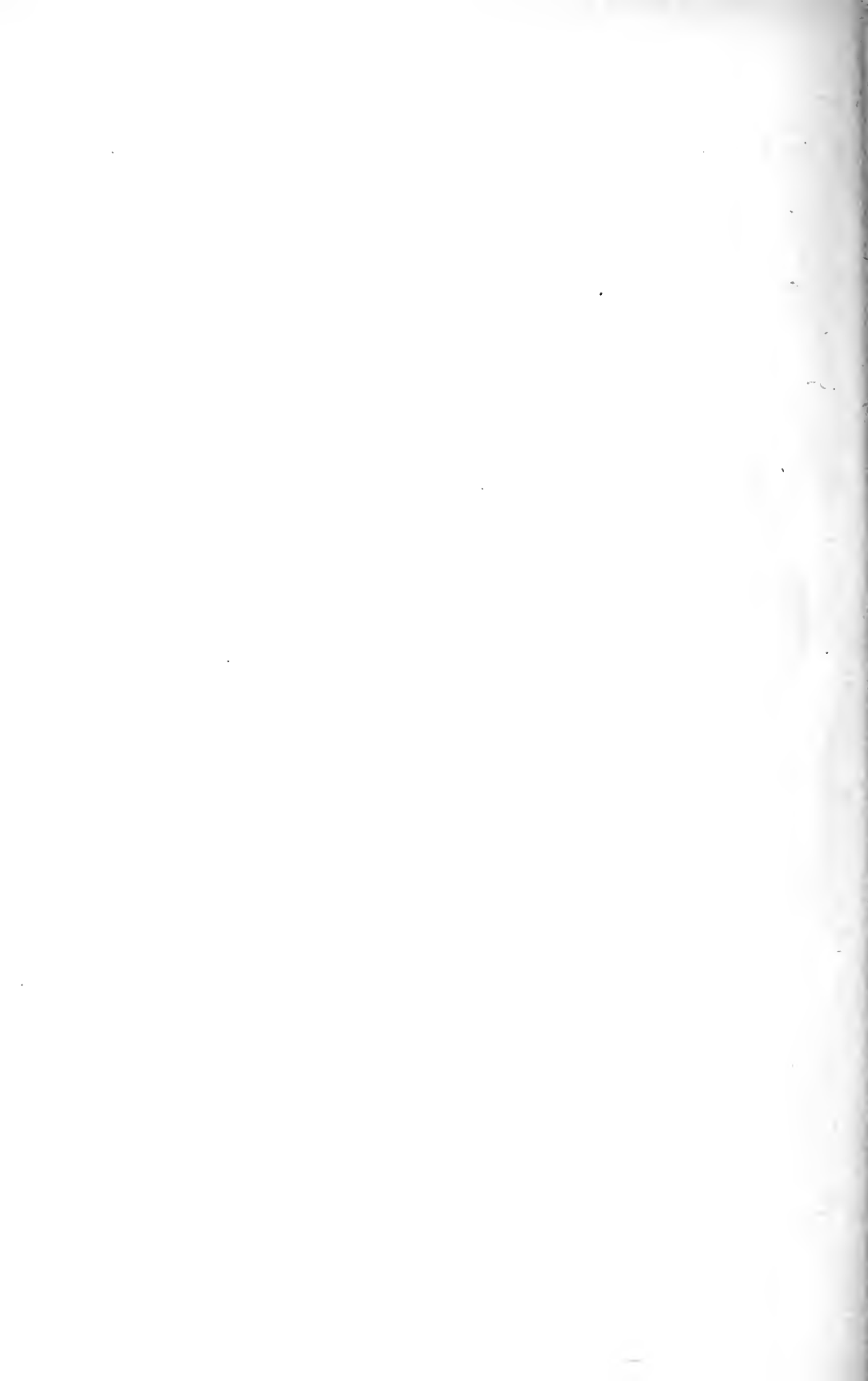
## PLATE 5.

FIG. 3. Camera lucida drawing. Leitz oc. 2, obj. 6. The figure is from the kidney of the carbonate animal of Experiment 11, Table I. The glomerulus is partially compressed by the swelling of the tubular epithelium. The capillaries are not distended with blood. The epithelium of the convoluted tubules, *a*, is severely swollen. The epithelium of the junctional tubules, *b*, fails to show the swelling. The animal was not protected against Gréhan's anesthetic by the carbonate solution. Early in the experiment it became anuric and showed no diuretic effect from theobromine or from a 5 per cent solution of sodium chloride.

## PLATE 6.

FIG. 4. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the control animal of Experiment 12, Table I. The animal received 25 cc. per kilo of a solution of sodium chloride equimolecular with a 3 per cent solution of sodium carbonate. The glomerular capillaries are not filled with blood. The epithelium of the convoluted tubules at *a* shows a severe grade of swelling. At *b* the cells have become necrotic. The junctional tubules at *c*

have an epithelium which is not swollen and in which the nuclei stain normally. During the development of a state of anesthesia the animal became anuric and remained anuric throughout the experiment. No diuretic effect was obtained from either theobromine or a 5 per cent solution of sodium chloride.



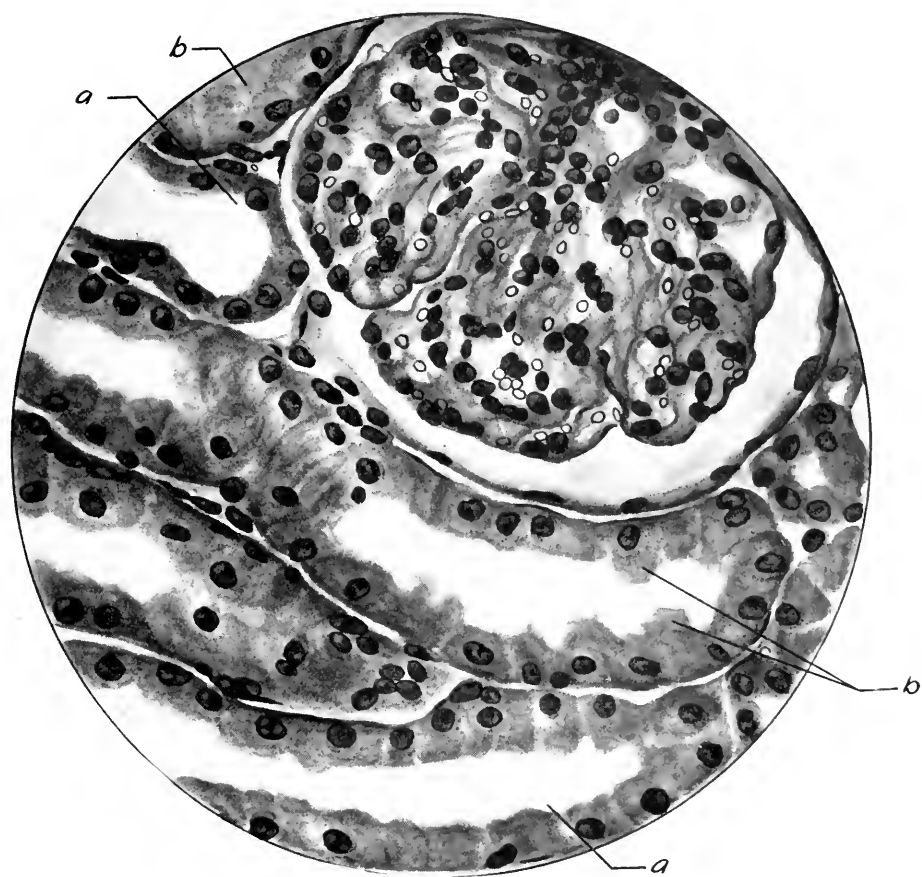


FIG. 1.

(MacNider: Toxicity of uranium. II.)

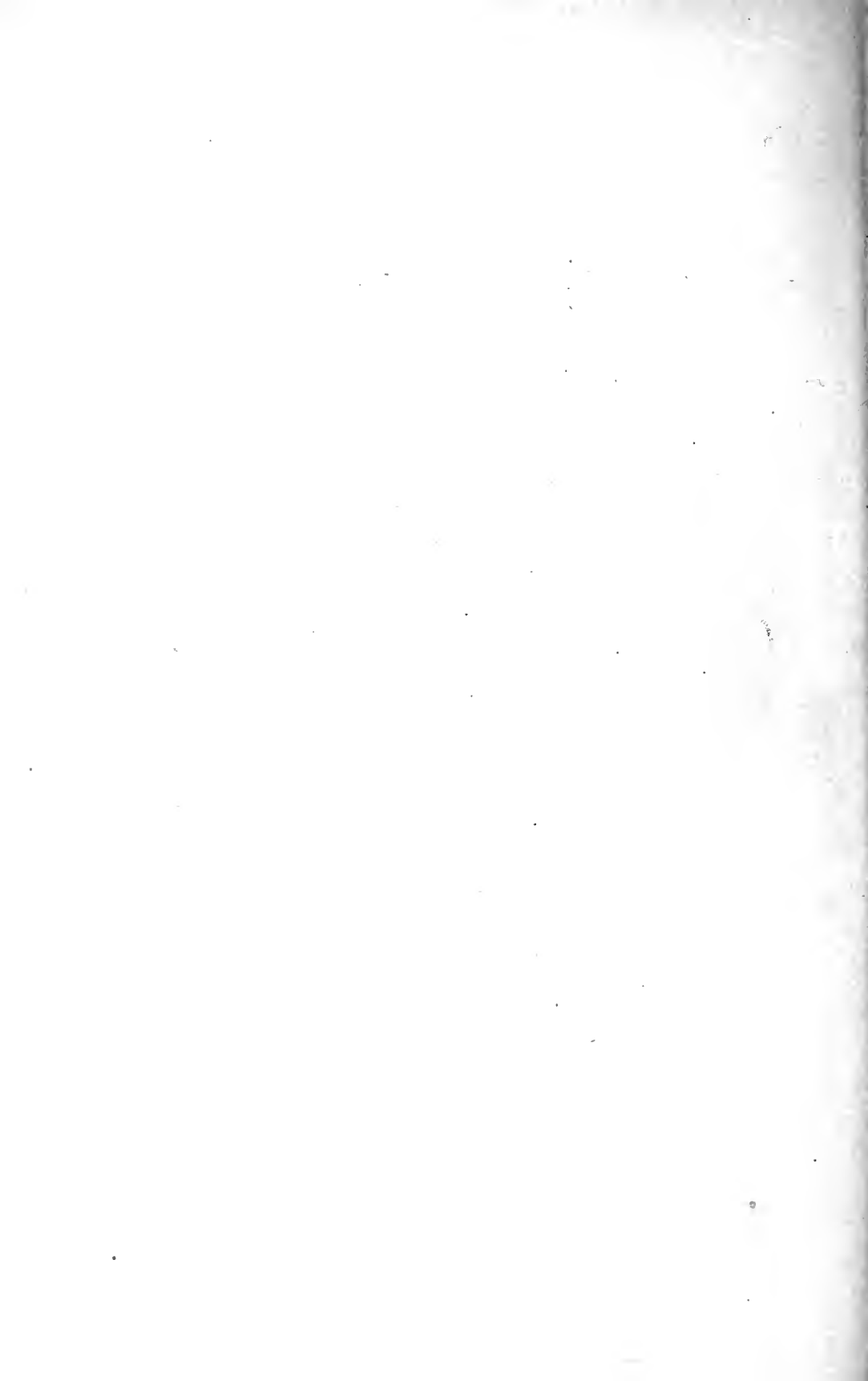




FIG. 2.

(MacNider: Toxicity of uranium. II.)





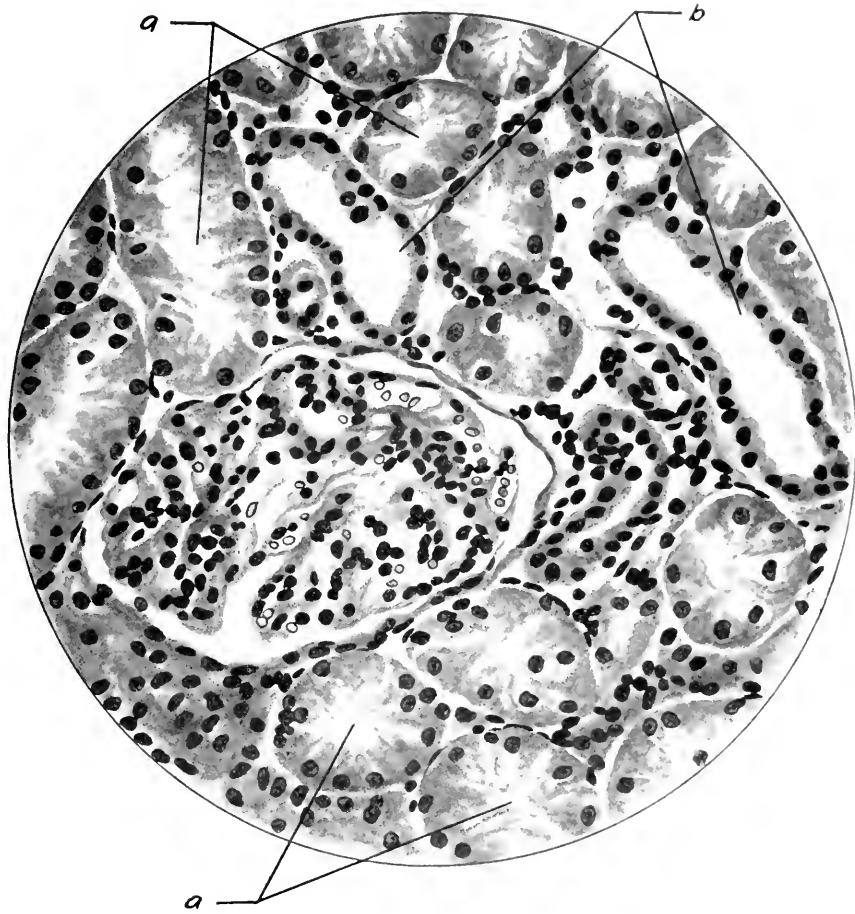


FIG. 3.

(MacNider: Toxicity of uranium. II.)



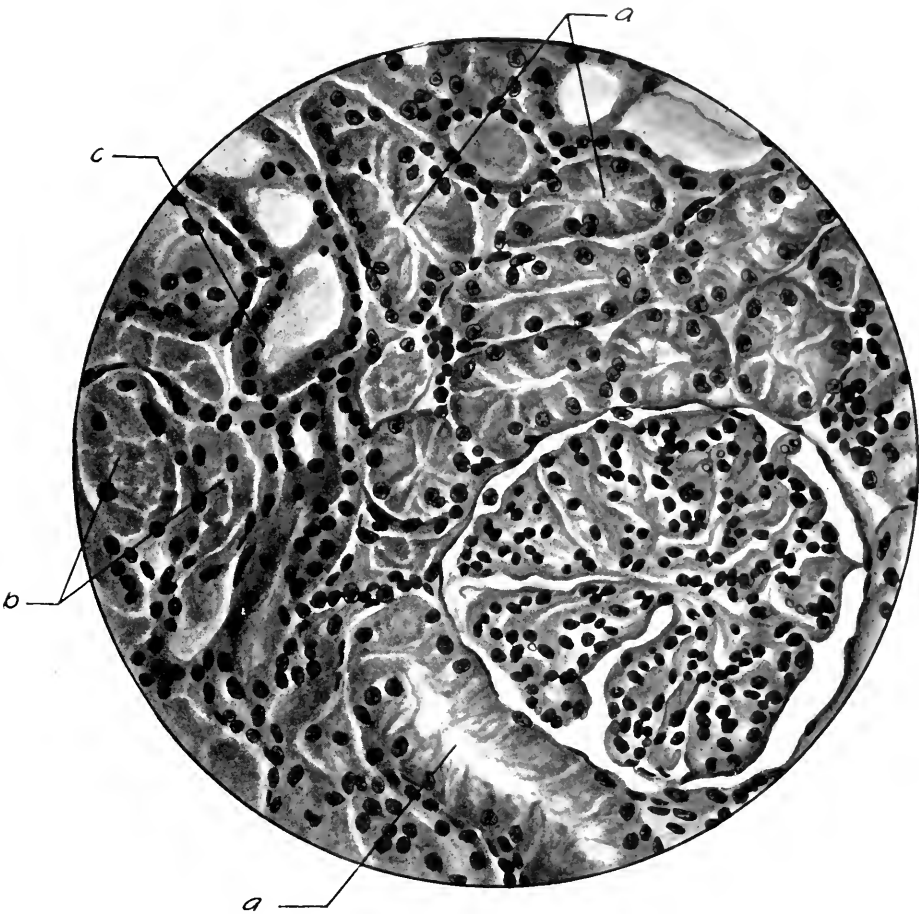


FIG. 4.

(MacNider: Toxicity of uranium. II.)



## CULTIVATION OF SPIROCHÆTA OBERMEIERI.

By HARRY PLOTZ, M.D.

(From the Pathological Laboratory of Mount Sinai Hospital, New York.)

PLATE 7.

(Received for publication, February 24, 1917.)

Up to the present *Spirochæta obermeieri* has not been grown from the blood of persons suffering from relapsing fever, nor has a spirochete of any species, as far as I have been able to discover, been cultivated directly from the blood of human beings. In this communication I wish to report the successful cultivation of *Spirochæta obermeieri* directly from the blood of patients suffering from European relapsing fever.

The studies were carried on in Serbia in the winter of 1915. The successful cultures, five in all, were made from cases clinically typical of European relapsing fever occurring in civilians and soldiers then residing in Macedonia.

Noguchi,<sup>1</sup> following his successful experiments on the cultivation of other spirochetes, succeeded in growing *Spirochæta obermeieri* from mice infected with relapsing fever. This spirochete had previously been kept alive by continued passage from animal to animal for a considerable length of time. The question arose whether spirochetes thus propagated for a long time become more amenable to artificial cultivation.

The method employed is the same as that used by Noguchi.<sup>1</sup> All cultures were taken during the febrile period of the disease. After careful disinfection of the skin with alcohol and iodine a vein in the antecubital space is punctured and 10 to 15 cc. of blood are withdrawn. A 15 cc. syringe with a thick bored needle is employed. It is important to withdraw the blood slowly, because rapid withdrawal may so injure the organisms that they will not grow. The needle is then removed from the syringe and the blood is slowly introduced into an Erlenmeyer flask containing 10 cc. of a 1.5 per cent sodium citrate solution in 0.85 per cent sodium chloride. The flask is gently agitated and the contents are then ready for culture. Culture tubes measuring 1.5 by 20 cm. are used.

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<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.

Into each tube is placed a piece of fresh sterile rabbit kidney tissue. The kidneys are removed aseptically and divided into about four pieces. About 5 cc. of the blood and sodium citrate mixture are added to each of a series of tubes. To this are added 15 cc. of sterile ascitic fluid. The ascitic fluid should be clear, free of bile and blood pigment, and should have a specific gravity of about 1.020. Noguchi has shown that an ascitic fluid which forms a fibrin filament when it comes in contact with the blood is best; no preservative should be added to the ascitic fluid and it should not be passed through a Berkefeld filter. Before use the fluid is tested out aerobically and anaerobically. Half the tubes are layered with sterile liquid paraffin. The tubes are incubated at 37°C.

The cultures were observed over a period of 2 weeks, but no definite appearance of growth was noted in the medium. Occasionally a slight clouding at the lower part of the ascitic fluid appeared. The tubes not layered with liquid paraffin showed the best growth.

Although only one or two spirochetes could be found in films made before culture (Fig. 1), a distinct increase could be noted after 48 hours' cultivation. In this series the maximum growth appeared on the 5th day and then gradually decreased. Preparations made from the junction of ascitic fluid and blood revealed masses and clumps of spirochetes. Preparations made from the upper part of the medium showed clumps and masses of spirochetes, but not so many as from the lower part (Figs. 2 and 3).

In young cultures the spirochetes appear shorter and thinner, but in older cultures they gradually become thicker and longer. The most striking appearance in young cultures is the spirochetal node which appears in practically every organism. These nodes are small thickenings which occur in the body of the spirochetes. From one to four may occur in the same organism. As the cultures become older these nodes become less numerous. I believe that these nodes probably represent the points where longitudinal division occurs.

Transplants are made about the 5th day by withdrawing 0.5 cc. of ascitic fluid from the lower part of the medium, without blood. This is inoculated into a similar tube as described above, except that 2 cc. of defibrinated human blood are added to each tube. The presence of hemoglobin aids the subsequent growth. In this manner *Spirochæta obermeieri* have been transplanted for five generations in two cultures.

EXPLANATION OF PLATE 7.<sup>2</sup>

FIG. 1. *Spirochæta obermeieri*. Blood film.

FIG. 2. Preparation from the upper part of the culture.

FIG. 3. Preparation from the lower part of the culture.

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<sup>2</sup> The microphotographs were kindly made by Professor Julius Rothberger of the Sero-Therapeutic Institute, Vienna.





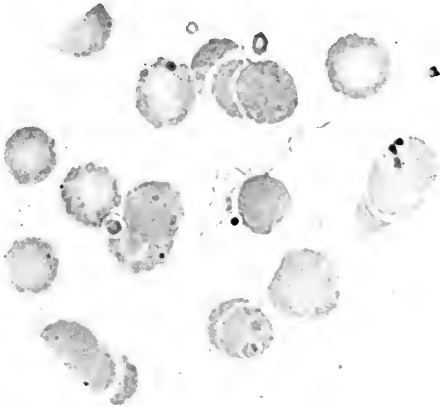


FIG. 1.

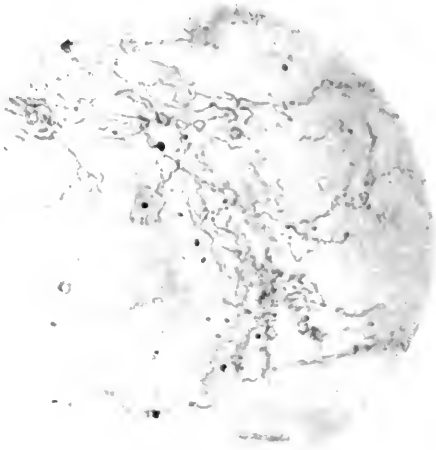


FIG. 2.

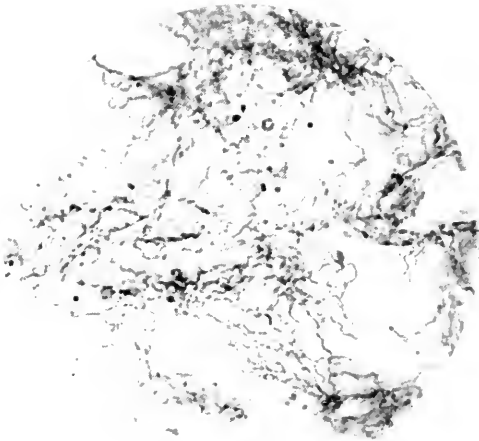
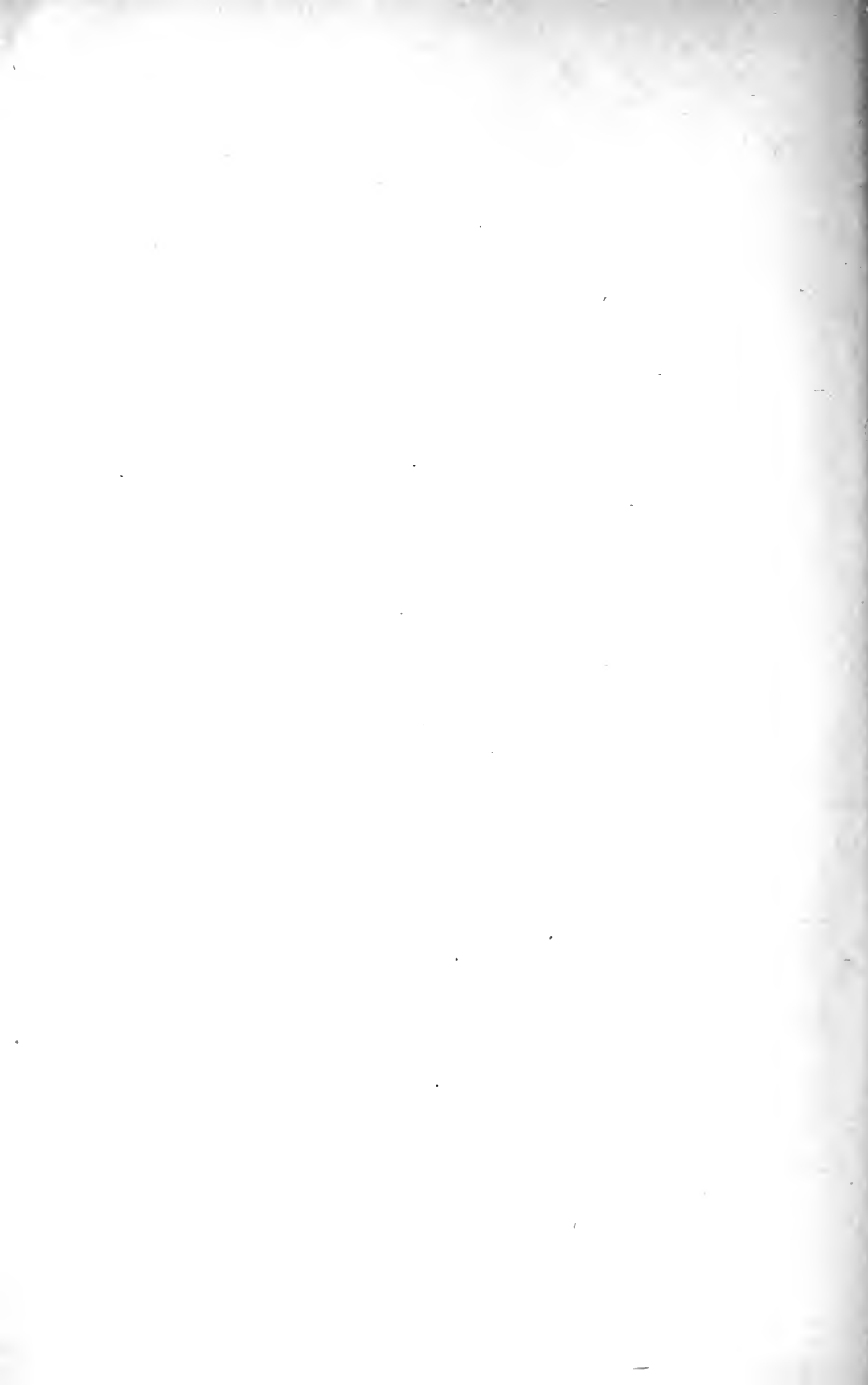


FIG. 3.

(Plotz: Cultivation of *Spirochaeta obermeieri*.)



## A VALVE TO REGULATE THE DELIVERY OF AIR AND ETHER VAPOR IN ANY PROPORTION.

By FREDERICK L. GATES, M.D.

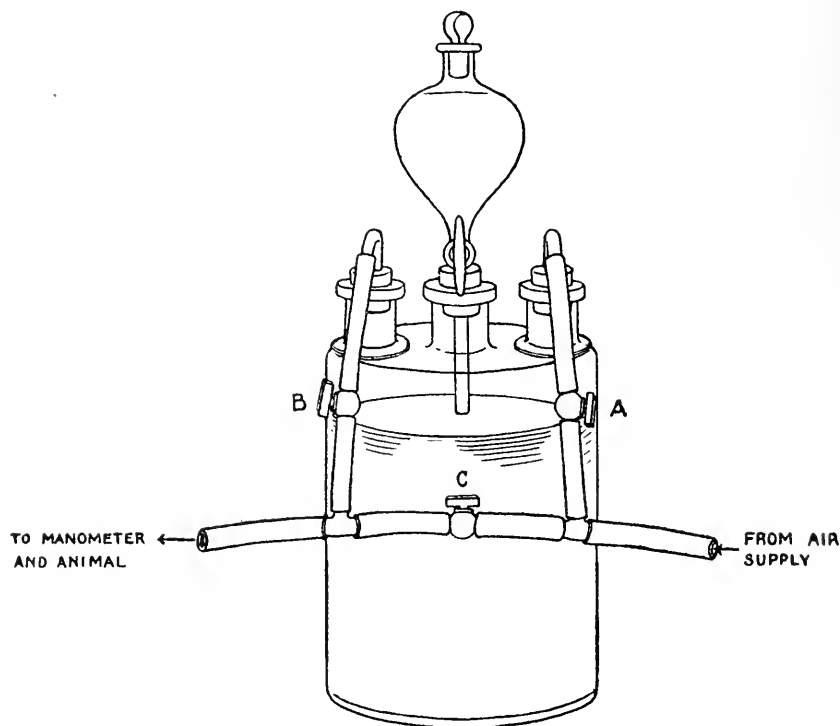
*(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)*

(Received for publication, March 5, 1917.)

Since the introduction of the Meltzer-Auer method of intratracheal insufflation for artificial respiration and anesthesia in 1909, this method of delivering air and ether vapor under positive pressure at the bifurcation of the trachea has been widely adopted in laboratories of research and in the surgical clinic. The close regulation of air pressure and ether vapor tension, the constancy of delivery independent of the respiratory movements of the anesthetized subject, and above all, the safety and surety of the method in supporting life in spite of respiratory failure, combined with smoothness of anesthesia and freedom from danger of aspiration pneumonia, make it the most satisfactory method for human as well as laboratory surgery.

An extensive literature has grown up in this country and abroad which by its character attests the success of the method in principle and practice. Most of the authors agree on the correctness of the principles involved; most of them have some contribution to make in the way of new apparatus for the simplification and perfection of the technique or the mechanics of the method. Many types of respiration machines have been described, their variety proving the wide flexibility of the method and its adaptability to individual preference and need.

Dr. Meltzer has always insisted upon the utmost simplicity in apparatus, and in his laboratory a consistent attempt has been made to reduce the mechanics of intratracheal insufflation to the lowest terms consonant with efficiency. In this way mistakes and complications may be avoided and the mind of the operator freed from constant supervision of the respiratory apparatus and the anesthesia.



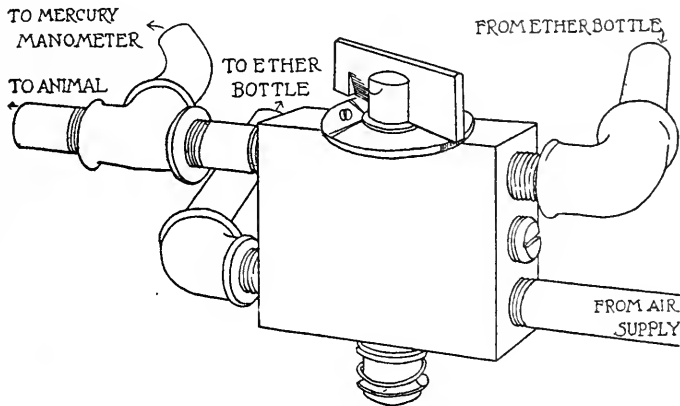
TEXT-FIG. 1. Woulfe bottle with the usual arrangement of tubing and stopcocks for air and ether vapor control. One-fourth the actual size.

#### *Method of Obtaining Ether Vapor.*

The simplest and most reliable method of obtaining ether vapor for insufflation anesthesia is the passage of the part of the air stream over the surface of ether in a Woulfe bottle (Text-fig. 1). The level of the ether is maintained by an auxiliary supply in a separating funnel. This is the method long in use in Dr. Meltzer's laboratory and has been adopted, with modifications, in several of the respiration machines intended for use in the clinic. If the ether surface is ample, it is not necessary to bubble air through the ether, or to warm the ether to promote evaporation, both of which are questionable procedures not unattended with danger.

Heretofore the diversion of a portion of the air stream over the

ether surface has been accomplished by means of stop-cocks in the tubing or some equivalent arrangement (Text-fig. 1). Three cocks are necessary for proper regulation of the ether supply. A and B control the air current in the ether circuit, while C regulates the passage of air unmixed with ether vapor. This arrangement has the disadvantage that any desired change in the proportion of ether vapor requires the manipulation of all three cocks to maintain the stream at its former volume and pressure, and even then it is practically impossible to determine the proportion of vapor-laden air obtained. It may be emphasized further that it is essential to have cocks A and B open to the same degree, lest an excess of ether vapor



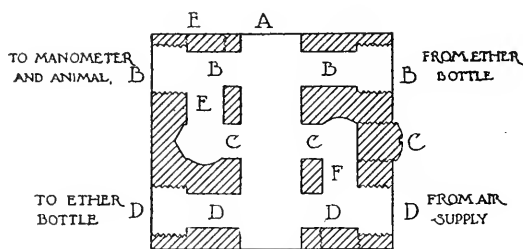
TEXT-FIG. 2. Constant volume valve, with fittings for the ether bottle and manometer. One-half the actual size.

be drawn into the tubing by the passage of air through C, or enter the system during the interruption of the air current if only one of the passages to the Woulfe bottle is subject to regulation.

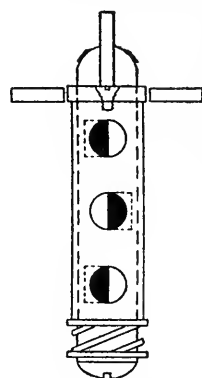
#### *Description of the Valve.*

The constant volume valve here described (Text-fig. 2) is in essence a convenient means of regulating all three stop-cocks synchronously, so that as the air stream over the ether is increased, the stream through C is reduced in exact proportion, and the sum of the volumes passing through the two limbs of the divided circuit remains constant. It consists of three channels corresponding to A, B, and C in Text-

fig. 1, but all contained in a single block, and all regulated synchronously by a single cock key through which the three channels are drilled. In the block (Text-fig. 3), D and B are the passages to and from the ether bottle, and C is the direct air passage in connection with D, F and E, B. As the passages B and D are closed by turning the cock key A, passage C must open in exact proportion so that the sum of the openings D (ether) and C (air) shall always equal a single opening of the same cross-section. This is accomplished by drilling the key so that the left edge of the holes corresponding to B and D are in line with the right edge of the hole corresponding to



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Longitudinal median section of the brass block, drilled and plugged. One-half the actual size.

TEXT-FIG. 4. Cross-section of the block near the key showing the arrangement of the square ports in the key in relation to the passages for air and ether vapor. One-half the actual size.

C. Text-fig. 4 shows a cross-section of the block close to the key, which is set for half ether, half air. Moreover, the holes in the key must be square, cutting off segments with the circular holes in the block, the sum of which equals the cross-section of a single channel.

The dial plate over which the handle of the cock key turns is calibrated to show the proportion of the air stream which is sent over the ether in the Woulfe bottle. It should be pointed out that this is not an absolute index of the tension of ether vapor obtained, as that is further dependent upon the rate of evaporation of the ether

and the rate of flow of the air stream. We have not found it necessary or desirable in ordinary anesthetics to determine the vapor tension of the ether with accuracy. Many complicating factors come into account which make a record of ether tension, either in millimeters of mercury or in percentage of ether vapor, merely an abstract number, useful only for the reestablishment of similar conditions at some later time if desired. This can be done with sufficient accuracy for ordinary purposes by resetting the key handle on the dial scale to the point previously determined. It should be emphasized that the condition of the patient is always the criterion of the amount of ether to be given. The anesthesia is to be regulated by observation of the patient, not by the ether vapor tension on a scale. Even in the laboratory, where there is less at stake than in the clinic, we find that different animals of the same species require different amounts of ether to produce safe anesthesia under apparently similar conditions.

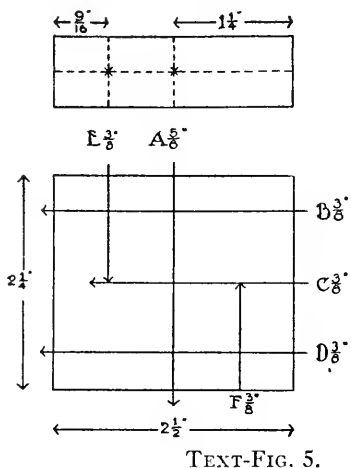
The valve described above has proved a simple and reliable means of regulating the ether vapor tension. Lately we have been employing this method of ether regulation for anesthesia in small animals, cats, rabbits, guinea pigs, rats, and mice, directing an ether-laden air stream to an ordinary cone, as used in the open drop method. The great advantages of intratracheal insufflation are not obtained, but the superiority over the open drop method of an unvarying supply of dry ether vapor, subject to close and instant regulation, is apparent. For one thing, the time of an assistant is saved.

#### *Directions for Constructing the Valve.*

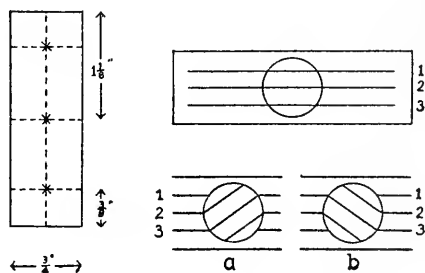
Since the valve can easily be turned out by a good technician or mechanic, working directions for its construction are appended.

The block is of brass,  $2\frac{1}{2}$  by  $2\frac{1}{4}$  by  $\frac{3}{4}$  inches. Text-fig. 5 shows the centers and directions for drilling the channels. Hole A,  $\frac{5}{8}$  inch in diameter, is for the key and should be drilled first, since it must be straight and true. Then B, C, and D,  $\frac{3}{8}$  inch in diameter, are drilled in turn, B and D through the block, C to within  $\frac{3}{8}$  inch of the opposite end. E and F,  $\frac{3}{8}$  inch in diameter, join B to C at one end of the block and D to C at the other. B and D are drilled out at each end and tapped for  $\frac{1}{4}$  inch pipe fittings. After the proper holes are

drilled through the key, C, E, and F are tapped out and plugs screwed in as far as F, B, and D respectively. The end of the plug in C may be cut as a screw head and used to regulate the size of F if the resistance of the passages to the ether bottle is found to be higher than that of the direct air passage C. The other plugs may be cut off flush with the block. A median longitudinal section of the block as drilled, tapped, and plugged is shown in Text-fig. 3.



TEXT-FIG. 5.



TEXT-FIG. 6.

TEXT-FIG. 5. Dimension drawing of the block as laid out for drilling. One-half the actual size.

TEXT-FIG. 6. Bottom of the block with lines for setting the key. a, position of the key when C is drilled through it. b, position of the key when B and D are drilled. One-half the actual size.

The valve key, to fit properly, should be turned down to  $\frac{5}{8}$  inch from larger stock and ground in with a very little emery and oil. A heavy grease used for lubrication will obviate any slight leak. The key is  $3\frac{1}{4}$  inches long. The valve handle, of strip brass 2 by  $\frac{3}{4}$  by  $\frac{1}{8}$  inches, is riveted in a slot in the key, the other end of which projects  $\frac{3}{8}$  inch beyond the bottom of the block and is fitted with washers and a spiral spring of moderate tension held in place by a screw or cotter-pin.

To drill the key properly, set the handle parallel with the long way of the block and draw three lines on the bottom of the block and key



as shown in Text-fig. 6. Line 2 is median, and lines 1 and 3 are parallel and  $\frac{3}{16}$  inch from 2. Turn the key until 2 on the key intersects 3 and 1 on the block (Text-fig. 6a). Drill C through the key. Then turn the key back until 2 on the key intersects 1 and 3 on the block (Text-fig. 6b), or, better, until the exact point is reached at which air will no longer pass through C. Then drill B and D through the key. This is the position of the key for full ether. Turning the key back to the first position, it will be discovered that C (air) is fully open and B and D are just closed. Intermediate positions of the key handle will give various mixtures of ether vapor and air. Square up the holes in the key with a small square file. A dial plate made of a large brass washer (Text-fig. 2) may be attached to the block by machine screws and calibrated mathematically or by test to show the proportion of ether-laden air in tenths of the total volume. As described, the valve is tapped for  $\frac{1}{4}$  inch pipe fittings as follows:

D, short pipe for tubing from the source of the air supply.

B and D', close nipple, elbow, short pipe for tubing to the ether bottle.

B', short nipple, tee, short pipes for manometer and for tubing to the animal.

The test of the valve's efficiency is that under working conditions it shall maintain a constant pressure in the mercury manometer when the valve handle is turned slowly between full ether and full air. If there is a rise in the pressure in the direction of full air, the resistance of the air circuit may be raised to that of the ether circuit by screwing in the plug C until it begins to cut off passage F.

#### SUMMARY.

A valve is described for the control of ether vapor for anesthesia which regulates the mixture of ether vapor from a Woulfe bottle with air in any proportion, without changing the volume or the pressure at which the mixture is delivered. The regulation of the air stream both to and from the ether bottle controls the mixture accurately and is an essential feature of the valve. Except for experimental purposes, it is not considered profitable to determine on an arbitrary scale the tension of the ether vapor obtained, because the depth of the anesthesia should always be judged by the condition of the subject.

The valve was devised for the Meltzer-Auer method of intratracheal insufflation, but it is adapted for use wherever a constant mixture of air and ether vapor is desired. Several respiration machines for supplying and interrupting the air stream, using this constant volume valve for the regulation of the ether supply, have been in use in Dr. Meltzer's laboratory for periods up to 2 years, and the simplicity and efficiency of the valve have been thoroughly tested. A description of the complete machine will be published later.

## THE RELATION OF MOSQUITOES AND FLIES TO THE EPIDEMIOLOGY OF ACUTE POLIOMYELITIS.

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(Received for publication, March 15, 1917.)

Of several hypotheses concerning the mode of conveyance of poliomyelitis, that of direct contact, supported by Flexner, appears to be the only one which stands on a substantial experimental basis.<sup>1,2</sup> Although in all epidemics instances have been reported in which more than two members of a family were victims of the disease, as a rule only one out of several children in a family is attacked. This peculiar feature of the epidemiology of poliomyelitis seemed to support the theory that the disease may be inoculated by the bite of an infected insect rather than transmitted directly from one individual to another.

Typical examples of insect transmission of disease are found in yellow fever, malaria, spirochetoses, trypanosomiases, and other tropical fevers. Rosenau and Brues<sup>3</sup> in 1912 announced their success in transmitting poliomyelitis from an infected to a normal *Macacus* monkey by means of the bites of stable-flies (*Stomoxys calcitrans*). Their experiment consisted in allowing many thousands of the flies to feed first on an infected monkey and immediately afterwards on a normal monkey. The transmission was mechanical, but not in the sense of an intermediary host. Anderson and Frost<sup>4</sup> confirmed Rosenau's finding in a

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<sup>1</sup> Flexner, S., The mode of infection and etiology of epidemic poliomyelitis, *Am. J. Dis. Child.*, 1915, ix, 353.

<sup>2</sup> Flexner, The nature, manner of conveyance and means of prevention of infantile paralysis, *J. Am. Med. Assn.*, 1916, lxvii, 279.

<sup>3</sup> Rosenau, M. J., and Brues, C. T., Some experimental observations upon monkeys concerning the transmission of poliomyelitis through the agency of *Stomoxys calcitrans*, *Tr. XVth Internat. Cong. Hyg. and Demography*, Washington, 1913, i, 616.

<sup>4</sup> Anderson, J. F., and Frost, W. H., Transmission of poliomyelitis by means of the stable fly (*Stomoxys calcitrans*), *Pub. Health Rep., U. S. Mar. Hosp. Serv.*, 1912, xxvii, 332.

limited number of instances, and then later failed to obtain further positive results. Howard and Clark<sup>5</sup> were unable to transmit the infection from an infected to a normal monkey by the bites of *Stomoxys calcitrans*. The peculiarity of conveyance of the infection is far from being solved by the theory of transmission by these blood-sucking flies.

The most plausible hypothesis so far advanced seems to be that offered by Amoss and Taylor,<sup>6</sup> who, having experimentally demonstrated the existence of a protective principle in the secretion of the nasal mucous membranes of normal individuals, consider that the incidence of infection may to a considerable extent depend upon the condition of these mucous membranes; that is, as long as they remain intact, the virus will be destroyed before it can become established in the individual. The disturbance of this natural protective mechanism exposes to infection, but not the presence or absence of the virus alone. They were able to show that the protective substance in question was more generally and abundantly present in adults than in young children.

The sudden disappearance of poliomyelitic cases with the return of cold weather is often used as an argument in favor of insect transmission of the disease. Flies and mosquitoes have constantly been under suspicion. While the elimination of these insects from our households is desirable as a matter of routine cleanliness, yet without a systematic experimental investigation they should not be accepted as a factor in the epidemiology of poliomyelitis. Indeed, it would be unwise if, through a misapprehension of the facts, the attention of health officers and laity alike were to be diverted from the real source of danger.

#### *Scope and Mode of Experiments.*

The question whether a given variety of insect plays a part in the spread of poliomyelitis is within reach of exact determination, since these insects are amenable to artificial propagation through many generations. During the past several months, we have been able to collect and propagate in tanks in the laboratories a quantity of *Culex pipiens* from sewer waters near Jersey City<sup>7</sup> and in

<sup>5</sup> Howard, C. W., and Clark, P. F., Experiments on insect transmission of the virus of poliomyelitis, *J. Exp. Med.*, 1912, xvi, 850.

<sup>6</sup> Amoss, H. L., and Taylor, E., Neutralization of the virus of poliomyelitis by nasal washings, *J. Exp. Med.*, 1917, xxv, 507.

<sup>7</sup> We are greatly indebted to Dr. Headlee, chief entomologist of the New Jersey Agricultural Experiment Station, at New Brunswick, and to Dr. Chidester

Queens County, Long Island. It was our intention to study several well known genera of mosquitoes at the same time, but owing to the lateness of the season, none of the *Aedes* or *Anopheles* species were available. The only variety which we were able to obtain was *Culex pipiens*. Experiments were also performed to determine whether the common house-fly or the bluebottle fly is able to take up the poliomyelitic virus and make it increase within its body.

There are at least two totally different ways of attacking the problem. The first method is to feed young growing larvæ with the active poliomyelitic virus by putting it in the polluted water in which they are being grown. When the adult mosquitoes have hatched out, they may be allowed to feed on a normal *Macacus* monkey. The second procedure is that ordinarily practised by investigators and consists in allowing the mosquitoes to feed first on an infected animal and then, within a certain length of time, on a normal animal.

The experiments with non-biting flies had to be carried out by still another method. They were allowed to feed on the nervous tissues containing an enormous amount of the poliomyelitic virus until they pupated. Part of the pupæ were allowed to hatch. Both the pupæ and the imagos were then crushed together and the emulsion was filtered through a Berkefeld filter. The clear filtrate was introduced intracerebrally and subsequent events were observed.

*Experiments with Mosquitoes Hatched in Polluted Water Experimentally Contaminated with the Virus of Poliomyelitis.*

To twelve jars, covered with a wire net cage and containing mosquito larvæ of various ages in ordinary stagnant water, were added varying amounts of the brain or cord emulsions or their filtrates derived from two monkeys that died of experimental poliomyelitis. The virus was introduced every 24 hours for a period of 10 days. The temperature of the laboratory was kept at about 70°F. and the

of Rutgers College, New Brunswick, for their assistance and instruction as to the propagation of mosquitoes in the laboratory. We wish to express our gratitude also to Mr. Winchell of Rutgers College, who assisted us in collecting the specimens in the field.

relative humidity at about 40 per cent. Numbers of adult mosquitoes, both male and female, hatched out from the jars every day. They were carefully collected and used for transmission experiments on normal monkeys. The rate of hatching from day to day is shown in Table I.

TABLE I.

Date.	Female.	Male.
<i>1916</i>		
Oct. 31	21	20
Nov. 1	12	9
" 2	11	2
" 3	17	8
" 5	10	12
" 6	15	8
" 7	30	18
" 8	25	9
" 9	13	18
Total.....	154	104

The first transmission experiment was performed with mosquitoes on two young *Macacus rhesus* monkeys. As the mosquitoes attack the monkeys only during the night, the experiment was begun at dusk and discontinued the next morning. The results were as follows: Of 150 females and 60 males put into the cage, 114 females were fully engorged with the blood, 23 females were apparently not engorged, and 52 males were still living. Some of the mosquitoes had been killed by the monkeys.

Close observation was maintained on the monkeys for 21 days, but no sign of poliomyelitis developed nor has any been noticed up to the present time.

Some of the engorged females sooner or later laid eggs and perished, while some died without oviposition. A number of female mosquitoes hatched out of the egg boats and also from the virus-containing water were kept alive for about 3 weeks and then allowed to bite two young *Macacus* monkeys. There were about 40 well engorged specimens when examined the following morning. The monkeys showed no symptoms of poliomyelitis.

These experiments demonstrate that the mosquitoes raised in water containing the virus of poliomyelitis in large quantities cannot transmit the infection by their bites to normal *Macacus* monkeys. There is no indication that the virus enters and multiplies in the body of mosquitoes, even if 3 weeks are allowed to elapse between the time of hatching and that of the biting experiment. The offspring of these females are not capable of transmitting the disease to the monkey.

*Experiments to Determine Whether the Females Well Engorged with the Blood of Infected Monkeys Will Ever Be Capable of Transmitting the Infection by Their Bites to Normal Macacus Monkeys.*

In order to decide this point, eight groups of mosquitoes, some obtained from the vicinity of Jersey City, and some from Queens County, Long Island, were allowed to feed on several *Macacus* monkeys inoculated for this purpose. To insure ourselves of including different stages of the infection, feedings were made every 24 hours after the inoculation of the virus into the monkeys and were extended into the paralytic stage, which of course varied in different animals from 6 to 9 days, according to the activity of the virus and the quantity injected. The protocols of these feeding experiments show the time which elapsed between the feedings and the transmission experiments. The term "feeding mosquitoes" is used merely to denote that these mosquitoes have sucked blood from an infected monkey.

Four out of the eight groups were tested on November 20; that is, 1 to 2 weeks after the time of feeding. The remaining four groups were allowed to bite after having been kept from 2 to 3 weeks. It must be mentioned that the engorged females do not survive for any length of time after oviposition, and comparatively few specimens remained alive in the second series of groups.

For the purpose of transmission two young *Macacus* monkeys were employed for each of the two experiments (Groups 1 to 4 and 5 to 8). We also inoculated 2 cc. of sterile normal horse serum into the intrathecal space in order to derange the protective mechanism which the meninges provide against the penetration of the virus

into the central nervous system in experimental poliomyelitis in monkeys. It may be recalled that Flexner and Amoss<sup>8</sup> demonstrated that a fraction of the virus, intravenously introduced, will readily localize in the central nervous system when the meninges are previously disturbed by the inoculation of various substances, although the monkey may bear a multiple of such doses with impunity if there has been no previous meningeal injury.

*Group 1. Feeding Mosquitoes.*—Nov. 13, 1916. Adult mosquitoes hatched in the laboratory from the larvæ collected in Queens were allowed to feed on *Macacus rhesus* 1 on the 4th day of experimental poliomyelitis. 30 mosquitoes were well engorged when examined the next morning. Used for experiments on Nov. 20, or in 7 days.

*Group 2. Feeding Mosquitoes.*—Nov. 15, 1916. Another lot of adult mosquitoes hatched in the laboratory from the same material were fed on the same monkey (No. 1) on the 6th day of the disease. The animal showed partial paralysis of the limbs. 23 well engorged females were obtained. Used for experiments on Nov. 20, or in 5 days.

*Group 3. Feeding Mosquitoes.*—Nov. 16, 1916. Another lot of adults from a similar source were fed on the same monkey (No. 1) on the 7th day of the disease. The animal was completely paralyzed. 17 engorged females were collected. Used for experiments on Nov. 20, or in 4 days.

*Group 4. Feeding Mosquitoes.*—Nov. 7, 1916. A lot of adults hatched in the laboratory from the larvæ from Queens were fed on *Macacus rhesus* 2 on the 9th day of the disease. 25 engorged females were obtained. Used for experiments on Nov. 20, or in 13 days.

*Group 5. Feeding Mosquitoes.*—Dec. 4, 1916. A lot of adult mosquitoes hatched in the laboratory from the larvæ secured from a Jersey City suburb were fed on two *Macacus rhesus* monkeys, Nos. 3 and 4, on the 7th day of experimental poliomyelitis. 13 engorged females were obtained. Used for experiments on Dec. 19, or in 15 days.

*Group 6. Feeding Mosquitoes.*—Dec. 1, 1916. A lot of adult mosquitoes hatched in the laboratory from the materials gathered in the neighborhood of Jersey City were fed on *Macacus rhesus* 5 on the 3rd day of experimental poliomyelitis. 34 engorged females were obtained. Used for experiments on Dec. 15, or in 14 days.

*Group 7. Feeding Mosquitoes.*—Nov. 24, 1916. A lot of adult mosquitoes hatched in the laboratory from the material from Queens were fed on *Macacus rhesus* 6 on the 2nd day of the disease. 45 engorged females were obtained. Used for experiments on Dec. 15, or in 21 days.

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<sup>8</sup> Flexner, S., and Amoss, H. L., The relation of the meninges and choroid plexus to poliomyelitic infection, *J. Exp. Med.*, 1917, xxv, 525.



*Group 8. Feeding Mosquitoes.*—Nov. 25, 1916. A lot of adult mosquitoes hatched in the laboratory from the material from Queens were fed on *Macacus rhesus* 7 on the 5th day of the disease. The animal had not yet become paralyzed. 20 engorged females were collected. Used for experiments on Dec. 15, or in 20 days.

The results of the experiments were negative, in spite of the fact that many females (about fifty) attacked the monkeys and became fully engorged with the blood sucked from them.

As in the earlier series of experiments, many of these engorged females oviposited and gave rise to a new generation of larvæ. It was easy to propagate them in the laboratory for an indefinite period. These offspring were in turn allowed to bite a normal monkey, but so far no positive transmission of the infection by their bites has resulted.

*Experiments with Non-Biting Flies Reared in the Laboratory with an Abundant Quantity of Poliomyelitic Virus.*

Non-biting adult flies may mechanically carry the virus of poliomyelitis just as they do various pathogenic microorganisms.<sup>9</sup> But no experimental evidence has been adduced to prove whether the virus can penetrate into the interior of the fly larvæ when the latter are fed on the poliomyelitic material, or, if it does, whether it is capable of multiplication within the body cavity of the larvæ. The question here is not the same as that of blood-sucking flies or mosquitoes, which may be the intermediary hosts as well as the direct transmitters of the virus by their bites. But in the case of non-biting flies, though the virus may multiply in their bodies, its distribution must depend upon their well known habits of gathering about food or persons while they regurgitate, resuck, and excrete. In this way the question of the non-biting flies as intermediary hosts of the virus would be quite as important as that of the biting varieties.

In the present experiment, a number of young larvæ of house-flies (*Musca domestica*) and of bluebottle flies (*Calliphora vomitoria*) were fed with the brain tissue of a *Macacus rhesus*, which had died of ex-

<sup>9</sup> Flexner, S., and Clark, P. F., Contamination of the fly with poliomyelitis virus. Tenth note, *J. Am. Med. Assn.*, 1911, lvi, 1717.

perimental poliomyelitis with typical symptoms and pathological findings. The activity of the material used was established in a subsequent experiment.

*Group 1.*—Nov. 23, 1916. About 50 house-fly larvæ were put on several slices of the fresh poliomyelitic monkey brain (No. 1). The larvæ eagerly penetrated into the nervous tissue as if trying to shield themselves from the light. At room temperature the tissue underwent a putrefactive decomposition within 24 hours, so the larvæ had to be placed on a fresh lot of the virus-containing brain. When left in the decomposed mass of tissue, many larvæ died. After being fed on the poliomyelitic brain for about 6 days, the larvæ became pupæ. After pupation, they were left with the brain tissue for several days longer and then washed from the tissue and removed to the refrigerator.

*Group 2.*—Nov. 28, 1916. Another lot of house-fly larvæ were similarly fed with poliomyelitic brain emulsion (No. 1) for 6 days. Pupation took place on Dec. 4. The pupæ were washed and removed to the refrigerator on Dec. 9.

*Group 3.*—Nov. 23, 1916. 25 larvæ of bluebottle flies were fed on several slices of brain tissue from a *Macacus rhesus* which had died of poliomyelitis. The larvæ attacked the tissue vigorously and within 24 hours seemed to have consumed a great deal of it. Unlike the house-fly larvæ, they feed on dead flesh or meat during their larval stage, but it was necessary to put them on fresh material every 24 hours in order to protect them from the deadly effect of the decomposed nervous tissue. Pupation occurred on Dec. 7. After several days, the pupæ were washed and removed to the refrigerator.

For the purpose of obtaining adult flies from the infected pupæ, a dozen specimens from each lot were put in a Petri dish and left at 80° F. Imagos hatched within about 12 days. These and the pupæ were used for the experiment described below.

*Preparation of Filtrate of the Infected Pupæ and Imagos of the House-Fly and the Bluebottle Fly.*

70 house-fly pupæ, Groups 1 and 2.	} Weight 1.36 gm.
12 " imagos, " 1 " 2.	
15 bluebottle fly pupæ, Group 3.	} " 0.97 "
6 " " imagos, " 3.	

These were mixed together and thoroughly ground up in a mortar, 22 cc. of 0.9 per cent saline solution being added. The milky emulsion thus obtained was briefly centrifuged at a low speed to separate the coarse particles from the fluid. The latter was then passed through a sterile Berkefeld filter V, the result being a clear and sterile filtrate.

*Animal Inoculation.*—A *Macacus rhesus* was inoculated intracerebrally with 3 cc. of the above filtrate on Dec. 16, 1916. The result was negative, no symptoms suggestive of poliomyelitis having been observed.

The foregoing experiment indicates that it is improbable that the virus of poliomyelitis is taken up by fly larvæ and multiplies therein. Not only does the virus fail to multiply, but probably it is rapidly destroyed within the body of the insects. The notion that these non-biting flies may act as intermediary hosts or a virus reservoir is not justified by the evidence brought out in the present study.

#### SUMMARY.

1. *Culex pipiens* raised from the larval stage in water experimentally contaminated with an abundance of poliomyelitic virus were found to be incapable of causing the infection when allowed in large numbers to bite normal *Macacus* monkeys.

2. *Culex pipiens* which were fed on infected poliomyelitic monkeys during different stages of the disease were found to be incapable of transmitting the infection when allowed in large numbers to bite normal *Macacus* monkeys. A previous disturbance of the meninges by an injection of horse serum into the intrathecal space did not alter the result, which was negative.

3. The offspring of the mosquitoes which were either reared in the infected tanks or fed on infected monkeys were found to be entirely harmless when allowed to feed in large numbers on a normal monkey. There was no hereditary transmission of the virus from one generation to another.

4. No trace of the virus of poliomyelitis was demonstrable in the filtrate of an emulsion of adult flies and pupæ of the common house-fly and bluebottle fly which were reared in the laboratory on slices, emulsion, or filtrate of monkey brain containing the poliomyelitic virus. The intracerebral injection of the filtrate produced no poliomyelitic infection in the normal monkey.



## NEW ANAEROBIC METHODS.\*

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### PLATE 8.

(Received for publication, March 12, 1917.)

The methods commonly used for the cultivation of anaerobic microorganisms are far from satisfactory. This applies to the partial anaerobes, such as the tetanus bacillus or *Bacillus botulinus*, but is particularly true of the absolute anaerobes, such as *Treponema pallidum* or the globoid bodies of poliomyelitis.

The vacuum jar described by Noguchi<sup>1</sup> in his method for the cultivation of *Treponema pallidum* has proved satisfactory in his hands, but requires a very strong desiccator, accurately ground, and a special type of vacuum pump. The American desiccators which I have obtained during the past 2 years, however, have either been so poorly ground that they would not hold a vacuum, or else they were so poorly made that they collapsed when subjected to vacuum.

After many trials, my difficulties were finally overcome through utilization of the suggestions of Laidlaw,<sup>2</sup> who used a catalyzer of oxygen and hydrogen in the preparation of anaerobic culture tubes. McIntosh and Fildes<sup>3</sup> developed the use of the catalyzer in making an anaerobic container, the "McIntosh bomb," but neither of these methods, useful as they are for the purposes for which they were devised, were suitable to our needs, and were therefore modified.

All the anaerobic methods to be described depend upon the catalytic action of platinized asbestos upon oxygen and hydrogen when they are brought into contact.

\*This work was done under the tenure of a William O. Moseley Travelling Fellowship from Harvard University.

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

<sup>2</sup> Laidlaw, P. P., *Brit. Med. J.*, 1915, i, 497.

<sup>3</sup> McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

*Methods for Anaerobic Test-Tubes.*

The simplest method is used in the cultivation upon agar slants of such relative anaerobes as the tetanus bacillus or *Bacillus botulinus* and is similar to the method described by Laidlaw. Platinized asbestos is first prepared in the usual way, or it may be purchased from any laboratory supply house. A small mass of the catalyzer is firmly fixed at the end of a platinum wire by coiling the wire about it. The other end of the wire is inserted into a short glass rod, and the rod is inserted into a No. 1 one-hole rubber stopper. The apparatus is wrapped in a package and autoclaved.

The water of condensation is removed from a plain agar slant, the tube inoculated, inverted, the cotton plug removed, and the tube filled with hydrogen by means of a sterile capillary pipette. The hydrogen may be obtained from a Kipp generator, or more satisfactorily from a hydrogen tank. It should be passed through a series of wash bottles containing silver nitrate, sulfuric acid, potassium permanganate, and lead acetate to remove all impurities.

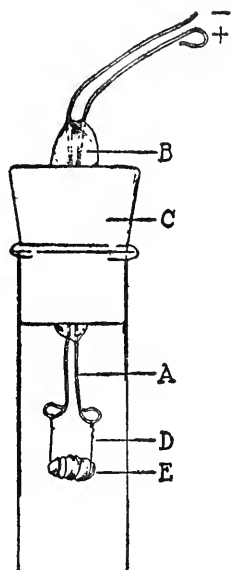
After allowing the hydrogen to fill the inverted inoculated test-tube, the platinized asbestos is heated for a moment in a free flame, the rubber stopper is inserted firmly into the inverted tube, and the end of the tube dipped into melted paraffin.

The catalyzer glows for a second or two as the hydrogen and oxygen are actively united, and the water formed is deposited upon the surface of the tube. The process is now complete, and the tube is ready for incubation.

This is essentially the method devised by Laidlaw and is very satisfactory for the growth of the usual anaerobes. The tetanus bacillus, for example, grows upon an agar slant in a thick, felt-like mass, in the profusion of its growth resembling a culture of *Bacillus subtilis*. It was necessary to devise a more strictly anaerobic method for our work, however, for Laidlaw's method could not always be relied upon to remove all traces of oxygen in the air, nor does it remove the oxygen from the surface of the media itself. The following method was devised to remove all the oxygen and has proved very satisfactory.

Two lengths of No. 22 nichrome wire, 6 cm. long, are separately

fused into a glass tube so that they are insulated (Text-fig. 1, A), and the glass tube, B, closed at each end, is passed through a one-hole rubber stopper, C. To the lower ends of the nichrome wire is attached a coil of fine (No. 31) nichrome wire, D, thus completing the circuit. In the coils of the fine wire is placed a small mass of platinized asbestos, E. The apparatus is placed in a package and autoclaved.



TEXT-FIG. 1. Sketch of anaerobic apparatus for the cultivation of absolute anaerobes in test-tubes.

A large test-tube, 20 by 1.5 cm. is used, to which 10 cc. of media are added, sterilized, and slanted. The water of condensation is removed and the tube inoculated. The tube is inverted, the cotton plug removed, and the tube filled with hydrogen by means of a sterile capillary pipette. The platinized asbestos mass, E, is heated for a moment in a free flame, and the rubber stopper, C, is then firmly inserted into the inverted tube and the end of the tube dipped in melted paraffin. The tube may now be placed in an upright position and sufficient electric current applied to the free ends of the wire to heat the fine nichrome wire wrapped about the platinized asbestos to a red heat. The catalyzer is thus heated, and the free

oxygen and hydrogen unite to form water. The tube is set aside for  $\frac{1}{2}$  to 1 hour, then the platinized asbestos is reheated in order to ignite any residual oxygen. The tube may now be incubated.

The method is very useful in growing all anaerobes, for the oxygen is always removed, whereas the Laidlaw method frequently fails. It is particularly useful for the cultivation of the stricter anaerobes. By this method the fusiform bacillus, for example, which is more strictly anaerobic than the tetanus bacillus, grows profusely in a thick, felt-like mass. By using this technique the organism of poliomyelitis grows upon ascitic agar slants, though much more slowly than other organisms; in fact, no growth is seen for 4 to 5 days, but at the end of 6 to 7 days, definite, tiny, raised, glistening colonies appear. These grow larger and larger, so that at the end of 12 to 14 days a definitely circumscribed, raised, opalescent colony is seen, some of these colonies even becoming as large as 1 mm. in diameter. In no instance was a growth of the globoid bodies of poliomyelitis obtained from the original material—brain and cord—but only from the fluid culture tubes of globoid bodies which had been growing in ascitic fluid media for several generations.

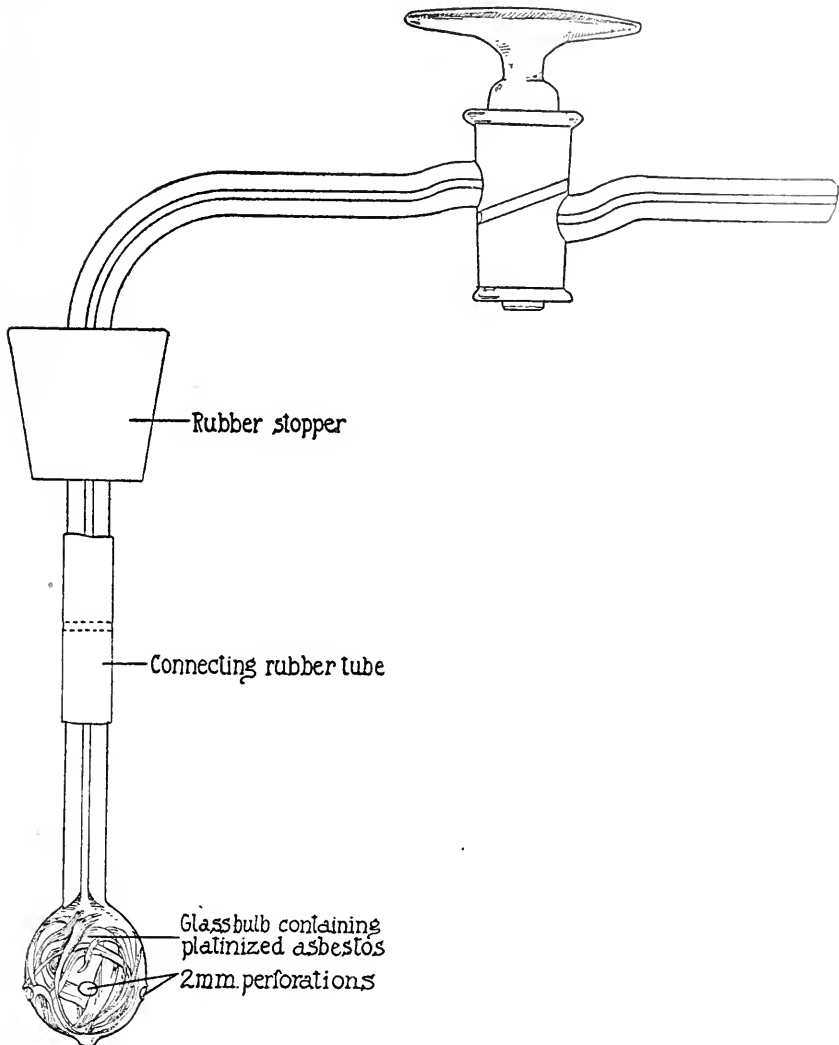
#### *Method for an Anaerobic Jar.*

The chief problem with which I was concerned, however, was the construction of an anaerobic jar, to be used as a container for a large number of tubes in the isolation of the organism of poliomyelitis from the infected brain and other tissues. All methods used for the cultivation of the globoid bodies failed in my hands until the jar described below was devised.

The jar used is an ordinary museum specimen jar (Fig. 1) about 30 cm. high and with an inside diameter of 12.5 cm. Two holes, 1.5 cm. in diameter, are ground in the cover, and into each hole is firmly inserted a No. 4 one-hole rubber stopper, carrying a ground glass "angle" stop-cock. To one of the stop-cocks is attached a rubber tube, at the end of which is a short piece of glass tube which reaches to the bottom of the jar. To the other stop-cock is attached, by a short rubber tube, a glass bulb, 2 cm. in diameter, which has been blown on the end of a capillary glass tube. The glass bulb is perforated with 5 to 6 holes, 2 mm. in diameter, and is filled with



platinized asbestos. The details of this apparatus are shown in Text-fig. 2.



TEXT-FIG. 2. Detail of the platinized asbestos bulb for the anaerobic jar.

Cultures are inoculated and placed in a glass tumbler, which is then placed in the jar, to which 100 cc. of a 10 per cent pyrogalllic acid solution have been added.

The glass bulb containing the platinized asbestos is heated over the free flame for a few seconds, and the cover is then cemented on. A rubber ring, 0.5 cm. thick, is placed between the jar and cover, all surfaces are cemented with Major's glass cement, and the metal clamp is screwed down with thumb and forefinger. The stop-cock to which the glass bulb is connected is placed on the vacuum pump, and gentle suction is applied for 2 to 3 seconds in order to insure a good initial flow of hydrogen and thus ignite the platinized asbestos at once. The stop-cock is now closed and attached to the hydrogen apparatus, and the gas is allowed to enter. This should be done carefully at first in order that an excess of hydrogen does not enter at once; for the gas should be burned as rapidly as it enters the jar. The platinized asbestos will soon be seen to glow and from this time hydrogen and oxygen will slowly unite, and the water formed will be deposited on the sides of the jar. When all the oxygen has united with the hydrogen, the platinized asbestos will become cool, but the hydrogen will continue to enter the jar until all the space formerly occupied by oxygen is replaced by hydrogen. The result is a hydrogen-nitrogen jar under approximately atmospheric pressure. The whole process should take about 15 minutes.

In order to have an index of the completeness of anaerobiosis the second stop-cock is connected with a bottle of 20 per cent sodium hydrate, freshly washed with hydrogen. By means of slight suction through the first stop-cock, 25 cc. of the sodium hydrate solution are drawn into the jar. Both stop-cocks are now closed, the ends sealed with cement, and the jar is incubated.

If the jar is satisfactory, the mixture of sodium hydrate and pyrogallous acid will remain colorless indefinitely. This solution should not be relied upon to absorb any remaining traces of oxygen, but is simply an indicator of the presence of oxygen, and if it becomes discolored, there has been a mistake in technique, and the jar is unsatisfactory; therefore the cover should be removed, and the process repeated.

#### *Method for Blake Bottles and Flasks.*

The use of the platinized asbestos in a perforated glass bulb has been applied to mass cultures of anaerobes in flasks or Blake bottles.

The Blake bottle is useful when large amounts of an anaerobic organism, such as the tetanus bacillus, are desired, and is particularly useful when a differential anaerobic plate is desired as, for example, in the isolation of anaerobes from the pus of infected wounds.

A ground glass stop-cock of the usual type is inserted through a No. 3 one-hole rubber stopper. A heavy glass bulb, 1 cm. in diameter, is blown at the end of the stop-cock, and five or six small perforations are made in the bulb. The bulb is then filled with platinized asbestos, and the whole apparatus autoclaved.

The Blake bottle is inoculated, the platinized asbestos heated in a free flame, and the rubber stopper tightly inserted into the bottle. Slight vacuum is then produced in the bottle by gentle suction, in order to insure a good flow of hydrogen. The stop-cock is connected with the hydrogen generator and the gas allowed to enter. The catalyzer glows for a few minutes, then cools, and in 5 minutes the oxygen has been replaced by hydrogen, and the process is complete.

The use of the perforated bulb has not proved satisfactory in single test-tubes because of the small air space.

The methods are simple, rapid, clean, and efficient. They are not expensive, for only a small amount of platinized asbestos is needed, and the material may be used repeatedly without deterioration. It would at first appear that there might be some danger of explosion in the jar, but such is not the case. The hydrogen ignites as soon as it enters the jar so that there is never an excess of hydrogen in the container. As the hydrogen and oxygen unite to form water, a slight vacuum is formed and this vacuum insures a continuous gentle flow of hydrogen until all the oxygen has been replaced.

The precautions to be taken are as follows: (1) Allow the hydrogen to enter the jar slowly. (2) Be sure that the hydrogen is catalyzed as it enters, as evidenced by the glowing of the asbestos. After the glow has once appeared the remainder of the process will continue gently and completely. (3) Do not disconnect the apparatus while the process is taking place, for there is a slight vacuum in the jar which has only partially been replaced by hydrogen, and if air is allowed to rush in over the catalyzer, there is a possibility of a slight explosion.

The results that have been obtained by the use of these methods, particularly the anaerobic jar, will be published in a subsequent paper.

## CONCLUSIONS.

1. Anaerobic methods have been devised which depend upon the catalytic action of platinized asbestos upon hydrogen and oxygen.
2. The methods may be utilized for the growth of anaerobes in test-tubes, upon Blake bottles, in flasks, and in a large container.
3. Because oxygen is so completely removed, the methods are of great value in the successful cultivation of absolute anaerobes.

## EXPLANATION OF PLATE 8.

FIG. 1. Anaerobic jar with platinized asbestos bulb.

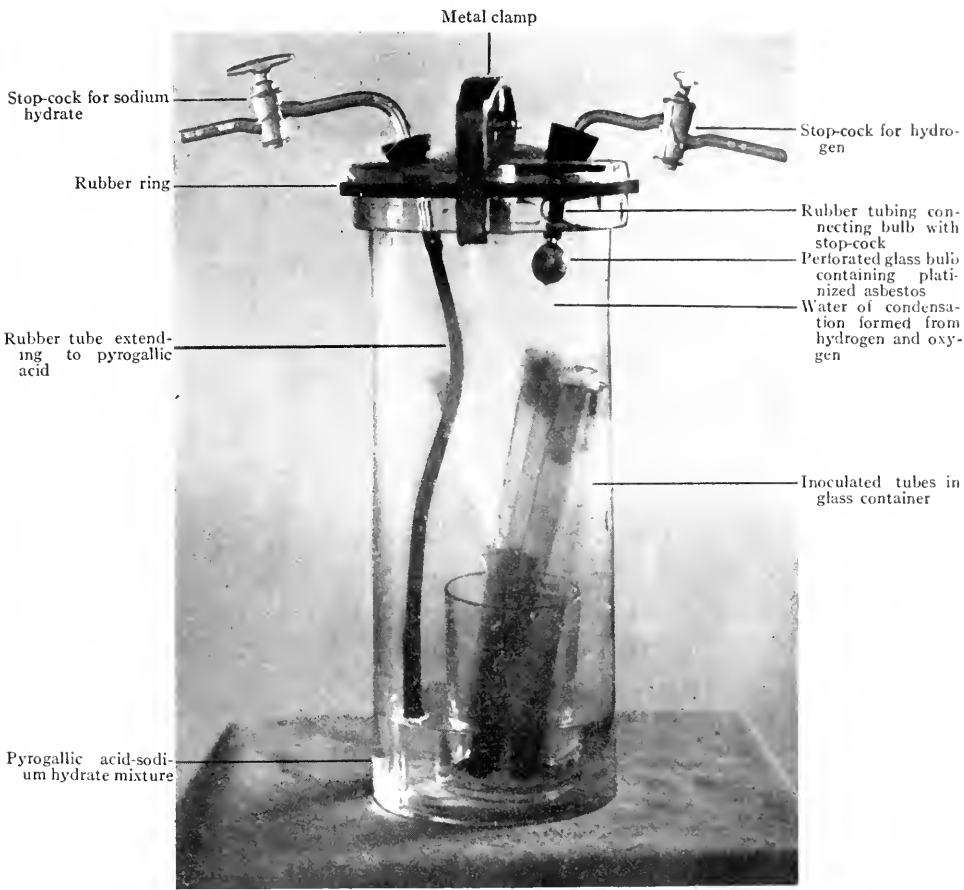


FIG. 1.

(Smillie: New anaerobic methods.)



## METHODS FOR THE DETERMINATION OF PNEUMOCOCCUS TYPES.\*

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It has been shown by Cole<sup>1</sup> and his associates that 75 to 80 per cent of cases of lobar pneumonia in adults are caused by three fixed types of highly parasitic pneumococci which can be differentiated readily by immunological methods, and that the remaining 20 to 25 per cent of cases are caused by a heterogeneous group of serologically independent varieties of pneumococci. These types have been classified by Dochez and Gillespie<sup>2</sup> primarily by means of animal protection experiments into Groups I, II, III (*Pneumococcus mucosus*), and IV (heterogeneous group). It has been shown that classification by agglutination corresponds to the classification by animal protection. Avery<sup>3</sup> has further shown that there is a considerable group of pneumococci which are related to Group II organisms in that mice are protected against infection with such atypical Group II pneumococci by Type II antipneumococcus immune serum and in that such pneumococci are agglutinable by Type II immune serum, though less rapidly and completely than Type II pneumococci. Immune sera prepared by the immunization of animals against such atypical Group II pneumococci, however, fail to agglutinate Type II organisms or to protect mice against infection with Type II strains. These atypical Group II pneumococci have been classified as Subgroups IIa, IIb, and IIx (heterogeneous). No such atypical strains have been encountered in Groups I or III. Statistical studies have shown that the mortality rate in pneumonia varies with the type of pneumococcus causing the disease, the percentage of deaths in each group being fairly constant from year to year. In a series of 400 cases<sup>4</sup> not receiving specific serum treatment the mortality rate was as follows: Group I, 25.2 per cent; Group II, 28 per cent; Group III, 56 per cent; Group IV, 14 per cent. These facts have be-

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\* This work was done under the tenure of a William O. Moseley Travelling Fellowship from Harvard University.

<sup>1</sup> Cole, R., *Arch. Int. Med.*, 1914, xiv, 56; *N. Y. Med. J.*, 1915, ci, 1, 58; *Tr. Cong. Am. Phys. and Surg.*, 1916, x, 138.

<sup>2</sup> Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

<sup>3</sup> Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

<sup>4</sup> Moore, H. F., and Chesney, A. M., *Arch. Int. Med.*, 1917, xix, 611.

come a valuable guide in prognosis and have provided a basis for rational specific serum therapy in lobar pneumonia which at present has been successfully developed for cases of pneumonia caused by Type I pneumococci.

The determination of pneumococcus types in cases of lobar pneumonia is rapidly coming into extended use because of its value as a prerequisite for specific serum treatment and in the field of prognosis. It is essential that a reliable and standard technique for the determination of types should be used and that the method should be as rapid as possible in order that serum treatment, when indicated, may be instituted at the earliest possible moment.

The method in common use consists in the intraperitoneal injection into a mouse of a specimen of the patient's sputum. By this means a rapid growth of the pneumococcus is obtained while other secondary organisms are usually inhibited. After a suitable interval, varying in the individual case from 5 to 24 hours as determined by preliminary peritoneal puncture with a capillary pipette, the mouse is killed and the peritoneal exudate is washed out with 4 to 5 cc. of normal salt solution. The leukocytes and fibrin are removed from the peritoneal washings by centrifugalization at low speed; the supernatant bacterial suspension is decanted into a second centrifuge tube and whirled at high speed until the bacteria are thrown down. This supernatant fluid is discarded, and the bacterial sediment resuspended in normal salt solution. The type of pneumococcus present is then determined by macroscopic agglutination tests with undiluted antipneumococcus immune serum. At the time of mouse autopsy cultures of the peritoneal exudate and heart's blood are made in broth and on blood agar plates for subsequent confirmation of the determination of type made on the peritoneal washings.

Certain factors have interfered with the rapid determination of types by this method in an appreciable number of cases. The most frequent difficulty encountered has been the growth of secondary organisms in the peritoneal exudate together with the pneumococcus, notably *Bacillus influenzae* and less frequently *Micrococcus catarrhalis*, staphylococci, streptococci, and *Bacillus typhi murium*. When this occurs agglutination of the pneumococci present is either inhibited or markedly delayed, or spontaneous agglutination occasionally occurs in all tubes. A delay of 18 to 24 hours results until ag-



glutination tests can be made on pure cultures of the pneumococcus obtained from cultures made of the heart's blood at the time of mouse autopsy. When *Bacillus influenzae* or *Bacillus typhi murium* are present in the peritoneal exudate they are usually present also in the heart's blood cultures and further delay takes place through the necessity of fishing and culturing pneumococcus colonies from the blood agar plate.

A further difficulty which has been encountered recently with the agglutination technique commonly used has been the occurrence of a small number of strains of pneumococci properly belonging in Group IV which agglutinate in all three types of antipneumococcus serum. The character of the agglutination is less rapid and less complete than that which occurs with the fixed types of pneumococci, resembling the agglutination of Subgroup II pneumococci by Type II serum. This phenomenon of cross agglutination is one that might be expected from our knowledge of other bacterial groups and it in no way invalidates the classification of the parasitic pneumococci into sharply defined immunological groups. It rather indicates that with the use of undiluted serum of high agglutinin titer a zone of non-specific reaction is encountered. While only a relatively small number of pneumococcus strains exhibit the phenomenon of cross agglutination, it has been shown by the more delicate precipitin test that a limited zone of non-specific reaction exists for strains of pneumococci of all four groups. It is necessary to give but one protocol of a considerable series to demonstrate this (Table I).

TABLE I.

*Determination of the Zone of Non-Specific Precipitin Reaction, Using Pneumococcus Type II Antigen and Antipneumococcus Immune Sera I, II, and III.*

Type II antigen 0.5 cc.	1:100	1:500	1:1,000	1:5,000	1:10,000	1:50,000	1:100,000	1:300,000	1:500,000
Serum I (1:10) 0.5 cc.....	++	++	+	=	—	—	—	—	—
“ II (1:10) 0.5 “ .....	+++	+++	+++	+++	+++	++	+	=	—
“ III (1:10) 0.5 “ .....	++	++	+=	+	=	—	—	—	—
Normal horse serum (1:10) 0.5 cc....	—								

The reactions were read after 2 hours at 37°C. and over night on ice. Pneumococcus II antigen was prepared by drying *in vacuo* the washed pneumococci from an 18 hour 1,000 cc. bouillon culture. The dried bacterial bodies were taken up in salt solution (10 mg. per cubic centimeter) and the suspension was repeatedly frozen and thawed until a faintly opalescent fluid free from bacterial bodies was obtained. Dilutions of the antigen were made in 0.85 per cent salt solution.

From Table I it is evident that a zone of non-specific precipitation occurs with Pneumococcus II antigen in dilutions not greater than 1:10,000 and that when higher dilutions are used the reaction becomes specific. Similar results have been obtained with antigen prepared from strains of Pneumococcus Type I and Pneumococcus Type III. Group IV pneumococcus antigens show a precipitin reaction only within the limits of the non-specific zone.

To obviate these difficulties certain improvements in the technique for the determination of pneumococcus types have been developed and will be presented below. The preliminary steps are the same as in the commonly used method and will be given in detail for the sake of completeness.

#### *Inoculation of Mice with Sputum.*

*Collection of Sputum.*—Care should be exercised in the collection of sputum to obtain a specimen from the deeper air passages as free as possible from saliva. This can be done in practically all cases, even the most difficult, with a little persistence. The physician or nurse should personally superintend the collection of sputum inducing the patient to cough until a suitable specimen is raised, care being taken not to allow the patient to swallow the lung sputum. The sputum is collected in a sterile Esmarch dish or other suitable container and should be sent at once to the laboratory for mouse injection. When delay is unavoidable the specimen should be kept on ice during the interval.

*Microscopic Examination of the Sputum.*—Direct films are made from the sputum and stained by Gram's method, with 10 per cent aqueous saffranin as a counterstain, by Ziehl-Neelson's stain, and

by Hiss's capsule stain. This serves to give an idea of the nature of the organisms present and an indication of the source of the sputum. Suitable lung specimens are relatively free, in most instances, from contaminating mouth organisms. It is frequently possible to identify Type III (*Pneumococcus mucosus*) organisms when they are present, as they possess a very large distinct capsule staining by both Gram's and Hiss's methods.

*Mouse Inoculation.*—A small portion of the sputum, about the size of a bean, is selected and washed through three or four changes of sterile salt solution in sterile Esmarch or Petri dishes to remove surface contaminations. The washed sputum is then transferred to a sterile mortar, ground up, and emulsified with about 1 cc. of sterile bouillon or salt solution, added drop by drop, until a homogeneous emulsion is obtained that will readily pass through the needle of a small syringe. 0.5 to 1 cc. of this emulsion is inoculated intraperitoneally into a white mouse with a sterile syringe. The pneumococcus grows rapidly in the mouse peritoneum while the majority of saprophytic mouth organisms rapidly die off with the exceptions noted above, *Bacillus influenzae*, and occasionally *Micrococcus catarrhalis*, staphylococci, and streptococci. Pneumococcal invasion of the blood stream also occurs early. *Bacillus influenzae* like wise invades the blood stream if present; other organisms as a rule do not.

The time elapsing before there is a sufficient growth of the pneumococcus in the mouse peritoneum for the satisfactory determination of type varies with the individual case, depending upon the abundance of pneumococci in the specimen of sputum and the virulence and invasive power of the strain present. It may be from 5 to 24 hours, averaging 6 to 8 hours with the parasitic fixed Types I, II, and III. As soon as the injected mouse appears sick a drop of peritoneal exudate is removed by means of peritoneal puncture with a sterile capillary pipette, spread on a slide, stained by Gram's method, and examined microscopically to determine whether there is an abundant growth of the pneumococcus present. If there is an abundant growth of the pneumococcus present in pure culture the mouse is killed and the determination of type proceeded with. If the growth is only moderate or if other organisms are present in any quantity, further time must be allowed until subsequent examination of the

peritoneal exudate shows an abundant growth of the pneumococcus. It should be emphasized that undue haste in killing the mouse is time lost in the end.

*Mouse Autopsy.*—As soon as the mouse is killed or dies, the peritoneal cavity is opened with sterile precautions and cultures are made of the exudate in plain broth and on one-half of a blood agar plate. Films are made and stained for microscopic examination by Gram's stain and Hiss's capsule stain. The peritoneal exudate is then washed out by means of a sterile glass pipette with 4 to 5 cc. of sterile salt solution, the washings being placed in a centrifuge tube. Cultures are then made from the heart's blood in plain broth and on the other half of the blood agar plate.

#### *Determination of Type.*

*Agglutination Method.*—When the pneumococcus is present in pure culture in the peritoneal exudate the determination of type may be satisfactorily made by macroscopic agglutination tests as follows. The peritoneal washings are centrifugalized at low speed for a few minutes until the cells and fibrin contained in the exudate are thrown down. The supernatant bacterial suspension is decanted into a second centrifuge tube and centrifugalized at high speed until the organisms are thrown down. The supernatant fluid is discarded and the bacterial sediment taken up in sufficient sterile salt solution to make a moderately heavy suspension. The concentration of bacteria should be similar to that of a good 18 hour broth culture of the pneumococcus. This suspension is used directly for macroscopic agglutination tests, being mixed with immune serum in small test-tubes in equal quantities of 0.5 cc. each.

To obviate the difficulty that occasionally arises from the occurrence of Group IV strains that show cross agglutination in all three types of immune serum, the optimum dilutions of serum and the optimum incubation time that will surely identify all type strains and fail to give any cross agglutination reactions have been determined on a large series of strains.<sup>5</sup> The results are shown in Table II.

<sup>5</sup> This work applies only to the antipneumococcus immune serum prepared at the Hospital of The Rockefeller Institute for Medical Research.

TABLE II.  
*Determination of Pneumococcus Types by Agglutination.*

Pneumococcus suspension 0.5 cc.	Serum I (1:20) 0.5 cc.	Serum II (undiluted) 0.5 cc.	Serum II (1:20) 0.5 cc.	Serum III (1:5) 0.5 cc.
Type I.....	++	—	—	—
“ II.....	—	++	++	—
Subgroups II a, b, x.....	—	+	—	—
Type III.....	—	—	—	++
Group IV.....	—	—	—	—

Incubation for 1 hour at 37°C.

From Table II it will be seen that a 1:20 dilution of Type I serum, making with the addition of an equal amount of pneumococcus suspension a final dilution of 1:40, a 1:20 dilution of Type II serum making a final dilution of 1:40, and a 1:5 dilution of Type III serum making a final dilution of 1:10, serve to agglutinate Types I, II, and III pneumococci respectively and fail to show any cross agglutination reactions with strains belonging in Group IV. It will further be seen that with 0.5 cc. of undiluted Type II serum, as well as with the 1:20 dilution, pneumococci belonging to the various Subgroups II may be identified and rapidly differentiated from Type II pneumococci in that they show partial to complete agglutination in undiluted Type II serum, but not in the 1:20 dilution at the end of 1 hour's incubation at 37°C.

For the determination of types of pneumococci in the peritoneal washings such serum dilutions give the most satisfactory and clear-cut results. Five small test-tubes are set up as follows: Tube 1, 0.5 cc. of Serum I (1:20) + 0.5 cc. of bacterial suspension; Tube 2, 0.5 cc. of Serum II (undiluted) + 0.5 cc. of bacterial suspension; Tube 3, 0.5 cc. of Serum II (1:20) + 0.5 cc. of bacterial suspension; Tube 4, 0.5 cc. of Serum III (1:5) + 0.5 cc. of bacterial suspension; Tube 5, 0.1 cc. of sterile ox bile + 0.3 cc. of bacterial suspension to determine the bile-solubility of the strain for differentiation from the streptococcus. The tubes are incubated in the water bath for 1 hour at 37°C. Agglutination of Types I, II, and III pneumococci in such serum dilutions is practically always immediate in the homologous serum and no agglutination occurs in the heterologous sera.

Rapid clumping of the organisms is seen to take place and may be brought out clearly by gentle agitation of the tubes. For the identification of Subgroup II pneumococci incubation is necessary, such strains showing partial to complete agglutination in undiluted Type II serum at the end of 1 hour's incubation. If no agglutination occurs and the organism is bile-soluble, it is classified as a Group IV pneumococcus.

*Precipitin Method.*—It has been stated above that the determination of pneumococcus types by macroscopic agglutination tests with the peritoneal washings is interfered with when other organisms are present, with a resultant delay of 18 hours or more before the type of pneumococcus present can be established. To obviate this difficulty the following method has been devised. Dochez and Avery<sup>6</sup> have shown that the pneumococcus produces in broth cultures during the period of active growth a soluble substance which gives a specific precipitin reaction with the homologous antipneumococcus immune serum. It seemed probable that this soluble substance or precipitinogen would be present in the peritoneal exudate of the mouse in sufficient quantity to give a specific precipitin reaction with the homologous serum and such has proved to be the case. The method to be described is dependent upon this phenomenon.

The peritoneal exudate is washed out with 4 to 5 cc. of sterile salt solution by means of a sterile glass pipette and placed in a centrifuge tube. The peritoneal washings containing cells, fibrin, and bacteria are immediately centrifuged at high speed until the supernatant fluid is water clear. The supernatant fluid is then pipetted off, with care not to disturb the sediment, which is discarded, and is mixed in quantities of 0.5 cc. each with an equal amount of the antipneumococcus immune serum in a series of small test-tubes as follows: Tube 1, 0.5 cc. of Serum I (1:10) + 0.5 cc. of supernatant peritoneal washings; Tube 2, 0.5 cc. of Serum II (undiluted) + 0.5 cc. of supernatant peritoneal washings; Tube 3, 0.5 cc. of Serum II (1:10) + 0.5 cc. of supernatant peritoneal washings; Tube 4, 0.5 cc. of Serum III (1:5) + 0.5 cc. of supernatant peritoneal washings. An immediate specific precipitin reaction occurs in the tube contain-

<sup>6</sup> Dochez, A. R., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 75.

ing the homologous immune serum, the other tubes remaining clear (Table III). No incubation is necessary. Two tubes of Type II serum are used for the purpose of distinguishing between Type II pneumococci and members of the Subgroups II, the former giving a precipitin reaction in both tubes, the latter only in the undiluted Type II serum. A negative reaction in all tubes indicates a pneumococcus belonging in Group IV.

The method has been tested with a large number of strains and has been consistently positive and specific with pneumococci of Types I, II, and III and consistently negative with pneumococci of Group IV. The presence of other organisms together with the pneumococcus in the peritoneal exudate does not interfere with the reaction and other organisms than the pneumococcus produce no substance that might give a false positive reaction.

TABLE III.

*Determination of Pneumococcus Types by the Precipitin Method.*

Supernatant peritoneal washings 0.5 cc.	Serum I (1:10) 0.5 cc.	Serum II (undiluted) 0.5 cc.	Serum II (1:10) 0.5 cc.	Serum III (1:5) 0.5 cc.
Type I.....	++	—	—	—
“ II.....	—	++	++	—
Subgroups II a, b, x.....	—	+	—	—
Type III.....	—	—	—	++
Group IV.....	—	—	—	—

The results with Subgroup II pneumococci have not been so satisfactory. Reference to Table III will show that pneumococci belonging to these groups give a precipitin reaction with undiluted Type II serum but not with the 1:10 dilution, thereby being distinguished from Type II pneumococci. A number of Subgroup II organisms, however, have been encountered in which the peritoneal washings have failed to give a precipitin reaction with undiluted Type II serum. In the identification of the fixed parasitic types of pneumococci this occasional difficulty is of little practical importance from the point of view of treatment as there is at present no specific therapy for cases of pneumonia caused by pneumococci of the Subgroups II. For purposes of classification and statistics these organisms can be

readily identified subsequently when the organism has been obtained in pure culture.

In order to determine the shortest time after mouse inoculation in which a clear-cut positive precipitin reaction may be obtained and whether it occurs as soon as a satisfactory agglutination test can be made, a series of mice have been inoculated intraperitoneally with measured amounts of pure pneumococcus cultures and the mice have been killed at varying intervals. Simultaneous determinations of type have been made by both the agglutination and precipitin methods on each peritoneal washing. The results are shown in Table IV.

TABLE IV.

*Comparison of Agglutination and Precipitin Methods.*

Mouse.	Time killed after inoculation with 0.01 cc. of culture.	Series A, Type I.		Series B, Type II.		Series C, Type III.	
		Agglutination.	Precipitin.	Agglutination.	Precipitin.	Agglutination.	Precipitin.
	<i>hrs.</i>						
1	4	—	—	—	—	—	—
2	5	—	—	—	—	—	+
3	6	—	—	—	+	—	++
4	7	—	+	—	++	—	++
5	8	++	++	++	++	++	++

From these experiments it is evident that the agglutination method possesses no advantage in point of time over the precipitin method. The presence of the soluble precipitinogen in the peritoneal exudate in sufficient quantity to give a clear-cut precipitin reaction coincides in the case of Type I pneumococci with the earliest time when satisfactory agglutination tests can be made. With Type II pneumococci it occurs earlier and with Type III still earlier. This phenomenon exhibits an interesting parallelism with the capsular formation and virulence of the three types of pneumococci.

The precipitin method possesses the following distinct advantages. It is available as soon as satisfactory agglutination tests can be made; incubation of the tubes is unnecessary; it is not interfered with by the presence of other organisms in the exudate; it is specific and shows no cross immunity reactions; it is applicable to mice which



through unavoidable circumstances have been dead for some time before the determination of type can be made and in which autolysis of the pneumococci or postmortem invasion of the peritoneal cavity by other organisms has made the agglutination method impracticable. For these reasons it is recommended as the method of choice in all cases.

*Identification of Type III Pneumococci by Morphological and Cultural Characteristics.*—If Type III antipneumococcus immune serum is not available for diagnostic purposes Type III pneumococci may be identified in most instances by cultural and morphological characteristics. *Pneumococcus mucosus* is usually somewhat larger, rounder, and less lanceolate than other types of pneumococci. It possesses a large distinct capsule which stains readily with Hiss's capsule stain and usually retains the pink counterstain with Gram's method. The peritoneal exudate produced on mouse inoculation is usually quite mucoid and colonies on solid media are moist, mucoid, and spreading. It is always bile-soluble. These characteristics usually serve to differentiate Type III pneumococci from other types. Occasional strains of pneumococci which agglutinate in Type III serum, however, are encountered which do not show well developed mucoid characteristics and cannot be distinguished culturally from other types. Furthermore, Type II strains are occasionally met with that exhibit fairly well developed mucoid characteristics. For these reasons the identification of Type III pneumococci by morphological and cultural characteristics is not always absolute, and the diagnosis should be established by immunological methods when Type III serum is available.<sup>7</sup>

#### *Confirmation of Type.*

The determination of type on the peritoneal washings should be confirmed by macroscopic agglutination tests with a pure bouillon culture of the pneumococcus obtained from culture of the heart's blood at the time of mouse autopsy. The technique is the same as that employed in the agglutination tests on the bacterial suspension obtained from the peritoneal washings and should include a test for bile-solubility.

<sup>7</sup> Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1917, xxv, 629.

*Determination of Types of Pneumococci in Blood Cultures, Spinal Fluids, Empyema Fluids, and by Lung Puncture.*

*Blood Culture.*—The usual technique in routine blood cultures is carried out. From a positive bouillon blood culture 10 cc. are removed by pipette and centrifugalized at low speed to remove the blood cells. The supernatant fluid is pipetted off and the bacteria are thrown down by centrifugalization at high speed, the supernatant fluid is discarded, and the bacterial sediment is suspended in sterile salt solution. The pneumococcus type is then determined by macroscopic agglutination tests following the same technique described above.

*Spinal Fluid and Empyema Fluid.*—Cultures are made by the methods ordinarily employed in culturing fluids and the type of pneumococcus is determined when the culture has grown out by the use of the same technique as that applied to blood cultures. If desired, in addition to culturing spinal fluids, a portion of the fluid may be centrifugalized at high speed to throw down the pneumococci present, and the sediment, taken up in 1 cc. of sterile salt solution, inoculated intraperitoneally into a mouse.

*Lung Puncture.*—This procedure should be resorted to only when it is impossible to obtain a suitable specimen of sputum or a positive blood culture. Cultures are made in bouillon of the lung puncture material and the determination of type is made by the same technique as that employed in the case of blood cultures.

*Determination of Pneumococcus Types by Direct Sputum Culture.*

It was thought that the determination of types of pneumococci by the precipitin method might be possible by direct culture of the washed sputum in bouillon without resort to mouse passage. Tubes of bouillon were inoculated with specimens of sputum and incubated 6 to 8 hours at 37° C. The cultures were then centrifugalized at high speed until the supernatant bouillon was clear, and precipitin tests were made by mixing equal parts of the supernatant bouillon and immune serum. In a few instances sufficient precipitinogen had been produced by the growth of the pneumococcus in the culture to give a positive precipitin reaction with the homologous serum. This by

no means invariably occurred with all specimens of sputum, however, and seemed to depend largely upon the number of pneumococci present in the sputum and to some extent upon the type of pneumococcus. The number of experiments done was small and the results were not sufficiently satisfactory to make the method of practical use. It is possible that further experiments along these lines might develop a technique which would prove available for use as a routine method.

#### SUMMARY.

The determination of pneumococcus types in lobar pneumonia is of value in the field of prognosis and as a prerequisite for specific serum therapy. The method for the determination of types should be as rapid as possible and a standard technique should be employed. The most satisfactory method is by the intraperitoneal inoculation of a mouse with the patient's sputum, by which means a rapid and abundant growth of the pneumococcus is obtained and secondary organisms are rapidly eliminated in most instances. The diagnosis of type is made directly on the peritoneal exudate. Certain factors in the method commonly used have interfered with the rapid determination of types in an appreciable number of cases, notably the growth of other organisms in the peritoneal exudate together with the pneumococcus, and some confusion has arisen because occasional strains of pneumococci have been encountered that show cross agglutination reactions when undiluted immune serum is used. Such reactions have been shown to be due to a limited zone of non-specific immunity and they in no way invalidate the classification of the pneumococci into sharply defined immunological groups. The optimum dilutions of serum have been determined that will agglutinate all type strains of pneumococci and fail to cause any cross agglutination reactions when mixed with equal amounts of pneumococcus cultures and incubated for 1 hour at 37°C. They are a 1:20 dilution of Serum I, a 1:20 dilution of Serum II, and a 1:5 dilution of Serum III. For the diagnosis of Subgroup II pneumococci undiluted Type II serum is required.

To obviate the other difficulties of the method commonly used a new method for the determination of types has been devised. It

depends upon the fact that there is produced by the growth of the pneumococcus a soluble substance which is present in the peritoneal exudate of the mouse in sufficient quantity to give a specific precipitin reaction with the homologous immune serum. The precipitin method can be used in all instances in which the determination of types by the agglutination method is possible, and it possesses certain distinct advantages which make it available when the agglutination method is impracticable. It is of particular value as a time-saving device in those instances where the presence of other organisms together with the pneumococcus in the peritoneal exudate causes a delay of 18 hours or more before the type of pneumococcus can be definitely established. It is therefore recommended as the method of choice in all cases. If desired, both the agglutination and precipitin methods may be applied to the same specimen of peritoneal washings.

## CICATRIZATION OF WOUNDS.

### V. NEW MATHEMATICAL EXPRESSION OF CICATRIZATION.

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(Received for publication, November 24, 1916.)

A new formula with two equations gives in mathematical terms Carrel and Hartmann's law:<sup>1</sup> "the rate of cicatrization diminishes at the same time as the size but less rapidly."

In the time  $dt$ , the area cicatrized,  $dS$ , is proportional to  $S$ :

$$dS = -\lambda S dt \quad \text{or} \quad \frac{dS}{dt} = -\lambda S$$

$\lambda$  is positive and the formula shows that the rate,  $\frac{dS}{dt}$ , decreases with  $S$ .

By integration we get

$$\int \frac{dS}{S} = -\lambda \int dt$$

$$(1) \quad \text{Log}_e S = -\lambda t + \text{Log}_e S_0 \quad \text{or} \quad S = S_0 e^{-\lambda t}$$

where  $S_0$  is the initial area.

If the coefficient  $\lambda$  is constant, the law of cicatrization can be expressed by simple logarithmic formula.

The rate of cicatrization decreases less rapidly than the size; that is,  $\lambda$  is not constant and must increase slightly when the area decreases. In the time  $dt$  the variation of  $\lambda$ ,  $d\lambda$ , is proportional to  $\lambda$ :

$$d\lambda = \mu \lambda dt \quad \text{or} \quad \frac{d\lambda}{dt} = \mu \lambda$$

If  $\mu$  is positive, the equation indicates that  $\lambda$  increases because the derivative  $\frac{d\lambda}{dt}$  is positive.

<sup>1</sup> Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

By integration we get

$$\int \frac{d\lambda}{\lambda} = \mu \int dt$$

$$(2) \quad \text{Log}_e \lambda = \mu t + \text{Log}_e \lambda_0 \quad \text{or} \quad \lambda = \lambda_0 e^{\mu t}$$

where  $\lambda_0$  is the initial value of  $\lambda$ .  $\lambda$  is calculated by equation (2) and with this value of  $\lambda$  we can obtain  $S$  by the equation (1).

The two coefficients  $\lambda_0$  and  $\mu$  may be determined to make the values calculated and observed correspond.

The area at any time can be obtained immediately without calculating the intermediate areas.

## CICATRIZATION OF WOUNDS.

### VI. BACTERIOLOGICAL ASEPSIS OF A WOUND.

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(Received for publication, February 9, 1917.)

In a previous communication<sup>1</sup> it has been shown that even a slight infection prevents normal cicatrization. Experiments on cicatrization must be carried out on surgically aseptic wounds. The degree of asepsis can easily be obtained by the method already described.<sup>2</sup> Surgical asepsis differs widely from bacteriological asepsis. Disinfected wounds which unite by first intention still contain numerous bacteria, particularly *Micrococcus epidermidis albus*. However, the experiments carried out by Carrel and Hartmann have shown that the new method of wound sterilization frequently leads to bacteriological asepsis.

The object of the following experiments was to determine to what degree wounds irrigated with Dakin's solution or treated with chloramine paste become bacteriologically aseptic.

#### *Preventive Action of Dakin's Hypochlorite Solution and of Chloramine-T.*

In the first series of experiments we undertook to ascertain what quantity of Dakin's solution or of chloramine-T is necessary to retard or prevent the growth of staphylococcus. This preliminary experiment was necessary in order to determine whether the quantity of antiseptic taken from the surface of the wound at the same time as the secretions was strong enough to inhibit the growth of the bacteria contained in the secretions.

<sup>1</sup> Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

<sup>2</sup> Carrel, A., and Dehelly, G., *Le traitement des plaies infectées*, Paris, 1916.

To 5 cc. of broth are added 0.1 to 2 cc. of Dakin's solution or of chloramine-T solution. A drop of a 24 hour broth culture of staphylococcus is then added. The staphylococcus used was obtained from a case of suppurative arthritis of the wrist treated during the preceding 15 days with Dakin's solution. Tables I to IV give the results of the experiments. They show that the power of Dakin's solution to restrain growth becomes evident after 12 hours if more than 0.4 cc. is used. In the same way, 0.3 cc. of 1 per cent chloramine-T also retards the growth. This power is relatively weak. The amount of Dakin's solution or of chloramine-T necessary to prevent completely the growth of the staphylococcus in 5 cc. of broth is, therefore, relatively large. 2 cc. of Dakin's solution or more than 1 cc. of 1 per cent chloramine-T are necessary for sterilization of the media.

TABLE I.

*Antiseptic Properties of Dakin's Solution. First Test.*

In 5 cc. of broth.	Cc. of Dakin's solution.																	Control.
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	2.0		
<i>days</i>																		
1	+	+	+	+	+	+	Weak.	?	—	—	—	—	—	—	—	—	+	
2	+	+	+	+	+	+	+	+	+	+	—	+	—	—	—	—	+	
3	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	+	
4	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	+	

TABLE II.

*Second Test.*

In 5 cc. of broth.	Cc. of Dakin's solution.								Control.
	0.9	1.0	1.1	1.2	1.3	1.4	1.5	2.0	
<i>hrs.</i>									
6		-	-	-	-	-	-	-	Weak.
12		-	-	Weak.	-	-	-	-	+
24	+	-	-	+	Weak.	-	-	-	+
48	+	-	+	+	+	+	+	-	+



TABLE III.  
*Restraining Power.*

In 5 cc. of broth.	Cc. of Dakin's solution.										Control.
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	
<i>hrs.</i>											
6	+	Weak.	Very weak.	?	-	-	-	-	-	-	+
12	+	+	Weak.	+	Weak.	-	-	-	-	-	+
24	+	+	+	+	+	+	+	-	+	-	+
36	+	+	+	+	+	+	+	+	+	+	+

TABLE IV.  
*Antiseptic Properties of 1 Per Cent Chloramine-T.*

In 5 cc. of broth.	Cc. of chloramine-T.																Control
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	2.0	
<i>hrs.</i>																	
6	Weak.	Weak.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Weak.
12	+	+	?	-	-	?	-	-	-	-	-	-	-	-	-	-	+
18	+	+	Weak.	-	?	Weak.	-	-	-	?	-	-	-	-	-	-	+
24	+	+	+	?	+	+	?	+	-	Weak.	-	-	-	-	-	-	+
36	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	+
48	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	+
3 days.	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	+

These experiments show that the small quantity of antiseptic removed with the bacteria by the platinum loop does not affect the growth of the cultures described below.

#### *Bacteriological Examination of Surgically Aseptic Wounds.*

As soon as films taken on several consecutive days showed that no bacteria were present, cultures were made from different parts of the wound. Before taking the cultures the irrigation was suspended for 2 hours, and in cases in which the chloramine paste had been used, all trace of the paste was carefully removed with distilled water and the wound was swabbed with sterilized gauze. It is advisable when taking the films to scrape the surface of the wound lightly with a platinum loop, care being taken to avoid making the granulations bleed.

TABLE V.  
Results of Experiments.

Case No.	Wound.	Treatment.	Duration of treatment.	Result of culture.	Bacteria recovered.	Result.
			days			
1	Complicated fracture of right humerus.	Dakin's solution.	7	+	<i>B. pyocyaneus</i> and various cocci.	Closure and suture 6 days after culture. Healing <i>per primam</i> .
2	Flesh wound of right knee.	"	7	+	<i>Staphylococcus albus</i> and an unknown bacillus.	"
3	Flesh wound of left leg.	"	7	+	<i>Micrococcus epidermidis albus</i> and <i>B. pyocyaneus</i> .	"
4	Flesh wound of right thigh.	"	8	+	<i>Enterococcus</i> , <i>Staphylococcus albus</i> , and various diplococci.	Closure and suture 4 days after culture. Healing <i>per primam</i> .
5	Perforated wound with fracture of right humerus.	"				
	(a) External wound.		8	—		
	Anaerobic culture.		11	—		
	Subculture.		11	—		
	(b) Internal wound.		8	+	<i>Micrococcus epidermidis albus</i> .	Closure and suture on day of subculture. Healing <i>per primam</i> .
6	Complicated fracture of neck of left femur.	"	12	+	<i>Staphylococcus aureus</i> , <i>Micrococcus epidermidis albus</i> , and various diplococci.	Closure and suture 13 days after culture. Healing <i>per primam</i> .
7	Complicated fracture of right tibia.	"	42	+	<i>Staphylococcus aureus</i> and <i>albus</i> .	Closure and suture on day of culture. Sloughing on 2 cm. of the suture. Surface granulation.
8	Surface wound following an old fracture of tibia.	Dakin's solution	109	—		
	Anaerobic culture.	and	112	—		
	Subculture.	chloramine-T.	112	—		
	Second culture.		117	+	<i>Micrococcus albus</i> and bacillus like <i>Proteus vulgaris</i> .	

TABLE V—*Continued.*

Case No.	Wound.	Treatment.	Duration of treatment.	Result of culture.	Bacteria recovered.	Result.
			days			
9	Wound of dorsal surface of foot following an old fracture of fifth metatarsal. (Wound heavily infected; gaseous gangrene.)	Dakin's solution and chloramine-T.	109	+	<i>Staphylococcus aureus</i> , <i>B. pyocyaneus</i> , and an unknown bacillus.	Surface granulation.
10	Flesh wound of calf.	"	117	+	<i>Staphylococcus aureus</i> and <i>albus</i> and an unknown bacillus.	"
11	Wound of dorsal surface of foot above fracture of metatarsus. Anaerobic culture. Subculture.	"	37	—		"
12	Complicated fracture of fourth metatarsal. Anaerobic culture. Subculture. Second culture.	"	40	—		"
			40	—		
			23	—		
			26	—		
13	Open wound of leg after ligature of popliteal and suture of posterior tibial arteries.	Dakin's solution.	26	—		Closure and suture on day of culture. Healing <i>per primam</i> .
			26	—		
			32	—		
			20	+	<i>Micrococcus epidermidis albus</i> .	
14	Flesh wound of right arm. Anaerobic culture. Subculture.	"	8	—		Surface granulation.
15	Large wound with fracture of shoulder blade. (a) Upper part of wound. (b) Lower part of wound. Subculture.	"	11	—		After slight reaction, inflammation on upper border of wound, redness for 3 days, and small hematoma surrounding the suture.
			11	—		
			16	+	<i>Staphylococcus albus</i> .	
			16	—		
			19	—		

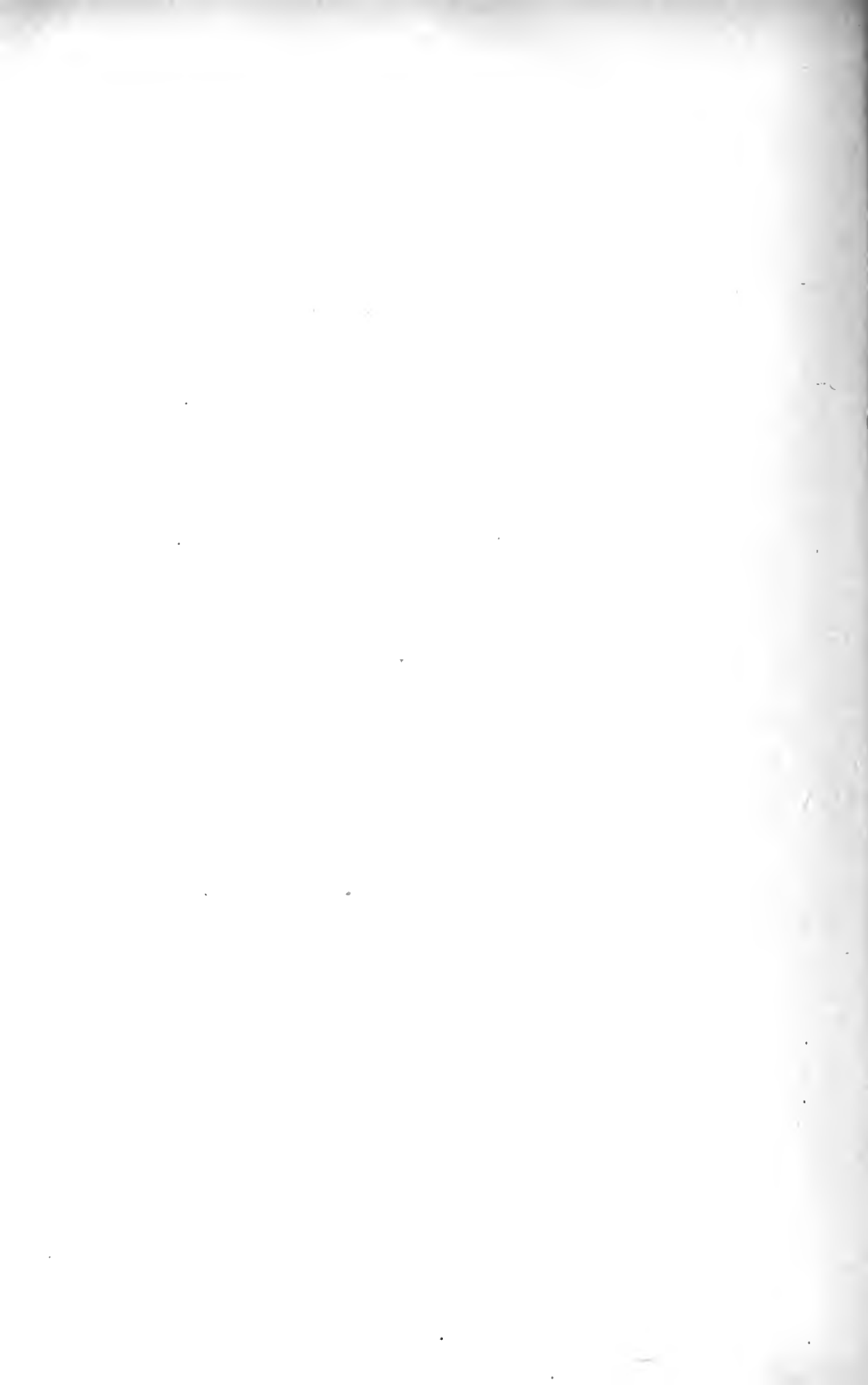
TABLE V—*Concluded.*

Case No.	Wound.	Treatment.	Duration of treatment.	Result of culture.	Bacteria recovered.	Result.
			days			
16	Flesh wound of calf with lymphangitis.	Dakin's solution.	19	+	<i>Micrococcus albus</i> and an unknown diplococcus.	Closure and suture on day of culture. Healing <i>per primam</i> .
17	Wound of left arm with comminuted fracture of humerus.	Dakin's solution and chloramine-T.	19	+	<i>Streptococcus</i> and <i>Staphylococcus albus</i> .	Surface granulation.
18	Flesh wound of right leg.	Chloramine-T.	20	—		"
	Anaerobic culture.		23	—		
	Subculture.		23	—		
19	Wound of left hand with complete destruction of fourth and fifth metacarpals.	"	23	+	<i>Staphylococcus aureus</i> and <i>albus</i> .	"
20	Wound of left knee following arthroto-my.	Dakin's solution	98	—		"
	Anaerobic culture.	and	101	—		
	Subculture.	chloramine-T.	101	—		
	Second culture.		110	—		

The tubes of broth were inoculated and incubated for 15 days at 36°C. If the culture remained sterile, a subculture was made on the 3rd day. At the same time, a fresh film was taken at the surface of the wound for an anaerobic culture in glucose broth with an upper layer of paraffin oil. Broth has proved an excellent medium for the bacteria found in wounds. Twenty cases were subjected to the usual treatment with Dakin's solution or chloramine paste (Table V).

## SUMMARY.

Of twenty infected cases treated with Dakin's solution or chloramine paste, seven were bacteriologically sterile. This proves that, in general, 35 per cent of the cases thus treated become bacteriologically aseptic. This degree of asepsis is not necessary in order to suture the wound, the absence of bacteria in films being sufficient. Complete sterilization of wounds, can, therefore, now be accomplished.



## CICATRIZATION OF WOUNDS.

### VII. THE USE OF CHLORAMINE-T PASTE FOR THE STERILIZATION OF WOUNDS.

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(Received for publication, February 9, 1917.)

It has been shown in a previous communication<sup>1</sup> that a wound cicatrizes rapidly if the surface is sterile. If it is more or less infected, the rate of cicatrization is slow or the wound enlarges. In order to obtain a convenient method for the sterilization of wounds we have endeavored to prepare an antiseptic paste which will retain its aseptic properties.

It has been found<sup>1</sup> that ointments and other fatty substances are inefficient when applied to wounds, because the bacteria and antiseptic are covered with fatty material which isolates them from each other and permits the bacteria to multiply freely. Hence the antiseptic paste must be soluble, and the bactericidal agent must be embodied in a substrate suitably chosen so that the whole constitutes a system physically homogeneous. On the other hand, to enable the antiseptic to act continuously the base should be absorbed slowly by the tissues in order to renew the surface of contact constantly. Neutral sodium stearate was used for this purpose because of the facility with which it is made antiseptic and also because it is not injurious to the tissues. It is well known that the slightly soluble sodium soaps, far from being irritating agents, are, on the contrary, soothing. Moreover, they give pastes sufficiently plastic for the dressing of wounds. One of Dakin's chloramines was selected as the bactericidal agent. After many trials we have used the following formula.

<sup>1</sup> Carrell, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

Neutral sodium stearate.....	86 gm.
Chloramine-T.....	4-10 "
Distilled water.....	1,000 cc.

Of the less soluble sodium soaps it is essential to choose those derived from saturated fatty acids and not having double ethylene linkages. The presence of such groups which readily take up the elements of hypochlorous acid ( $\text{HClO}$ ) causes a rapid disappearance of chloramine. On the other hand, stearic acid is a product of sufficient purity and is easily procured; its sodium salt obtained by boiling the calculated amount diluted with caustic soda is aseptic.

We have tried some pastes less concentrated in stearate, but they separate into two portions; the lower part is watery, and the upper portion richer in stearate so that it has the concentration indicated above, which is a minimum.

We have chosen as an antiseptic to combine with the sodium stearate one of the substances studied by Dakin, known as chloramine-T, which is the sodium salt of toluene sodium *p*-sulfochloramide. The reasons for choosing this substance were its high bactericidal power, the absence of caustic action on the skin, the possibility of an exact estimation of its strength, and its stability at a high temperature, which allows the substances to dissolve in a boiling solution of stearate. The question of using sodium hypochlorite was not considered because this product changes rapidly under the influence of heat, and especially because of the sensitiveness of soap solutions to the action of electrolytes.

Several trials were made with various proportions of chloramine-T. 20 gm. per kilo seem to make the paste irritating, thus rendering it useless. 15 gm. per kilo are tolerated by the wounds but are slightly irritating to the skin and congest the granulations, giving them a purple color. 10 gm. per kilo cause neither irritation nor pain; the wounds tolerate application for weeks. At this concentration the bactericidal action is strong enough to disinfect surface wounds completely, as rapidly as Dakin's solution (sodium hypochlorite 0.50 per cent). At the level of contact of the paste and tissues a thick greenish liquid gradually forms, which is apparently the result of the action of the secretion of the wound upon the paste. Bacteriological examination shows the secretion to be sterile, but it is



important to wash it away every day with neutral sodium oleate before making a fresh application. For wounds in the process of cicatrization, paste with 10 gm. of chloramine-T per kilo seems to retard the repair slightly. If the percentage of chloramine-T is further decreased, the antiseptic action decreases proportionately. With 4 gm. per kilo the action on infected wounds is extremely weak, but those that have been disinfected remain sterile and their cicatrization is normal.

The preparation of chloramine paste is as follows: Boil a liter of distilled water and add 80 gm. of stearic acid. When this has melted, gradually add enough caustic soda to saponify the fatty acid and after complete solution add 4 to 10 gm. of chloramine-T, according to the concentration desired. The mixture is then placed in a mixing machine and shaken until thoroughly cooled. The paste is a smooth snow-white cream. Microscopic examination shows that it is composed of a compact felting of fine needles retaining in capillary suspension a colorless liquid. The sodium stearate is slightly soluble at a cold temperature and produces this crystalline felting which retains in its interstices the antiseptic solution. The paste can be kept either in low glass jars or tin tubes, as tin is not corroded by chloramine-T.

The principal disadvantage of this paste is its poor power of preservation; numerous trials showed that 10 per cent of chloramine-T disappeared per month. Substances which might have rendered the paste more stable were either inefficient or lessened its keeping properties. The stability of the paste is limited by the stability of the solution of chloramine-T because the antiseptic is in solution in the paste.

#### CONCLUSION.

Dakin's toluene sodium *p*-sulfochloramide, mixed with sodium stearate, forms a paste sufficiently active and stable to be used in the treatment of wounds.



## CICATRIZATION OF WOUNDS.

### VIII. STERILIZATION OF WOUNDS WITH CHLORAMINE-T.

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(Received for publication, February 23, 1917.)

In a previous article<sup>1</sup> it was shown that the presence of bacteria at the surface of a wound retards the normal process of cicatrization. According to the nature and size of the infection, the curve representing cicatrization deviated from the calculated curve.<sup>2</sup> In order to investigate the substances which are capable of influencing tissue repair, it is, therefore, imperative that the wound should be kept in an aseptic condition. No specific influence on the progress of healing could be attributed to the substance experimented with unless the possible action of infection was entirely eliminated.

Sterilization of a wound is easily effected by the application of Dakin's hypochlorite solution at the surface of the tissues under appropriate conditions of concentration and duration.<sup>3</sup> In the experiments to be described, it was attempted to simplify the method by substituting for the instillations of Dakin's hypochlorite solution a paste designed gradually to yield up to the tissues one of Dakin's chloramines contained therein. Investigations were undertaken to ascertain whether this paste would be able to keep a sterile wound in an aseptic condition, as well as to sterilize an infected wound, and whether it would retard tissue repair.

#### *Chloramine Paste.*

Some unpublished experiments of Dakin had shown the necessity of avoiding fatty substances in the composition of paste intended for the sterilization of wounds.

<sup>1</sup> Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

<sup>2</sup> du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451.

<sup>3</sup> Carrel, A., and Dehelly, G., *Le traitement des plaies infectées*, Paris, 1916.

Dakin mixed with vaseline or lanoline certain antiseptic substances which are insoluble in water. Once these substances were applied to the wounds, their bactericidal power became almost negligible. Carrel also experimented with aqueous solutions of antiseptic substances mixed with lanoline, but they failed to have an influence on the infected wounds. It appeared that the active substance remained in the lanoline and exerted no effect upon the bacteria. Carrel next combined certain antiseptic substances in agar, but found that the consistency of the agar rendered it unsuitable for practical use. The cakes yielded to the tissues the liquid they contained, but they were too brittle and easily destroyed.

It was concluded from these experiments that, in order to be efficient, the bactericidal substance would have to be combined with a fat-free substance capable of being moulded exactly to the surface of the wound. At this point Daufresne<sup>4</sup> succeeded in preparing a paste composed of sodium stearate and of toluene sodium *p*-sulfochloramide, the bactericidal properties of which had been discovered by Dakin and which is named chloramine-T for convenience. This paste is sufficiently firm not to flow away when applied to a wound, and yet fluid enough to be moulded to the anfractuosités of a granulating surface or of a fractured bone. The paste contains 8 per cent of sodium stearate and 4 to 15 parts per 1,000 of chloramine-T.

The paste is placed in sterile glass receptacles from which it is withdrawn at the time of dressing by means of wooden spatulas which have been sterilized in the autoclave. It is also preserved in tin tubes, to the ends of which is attached a rubber tube ending in a tapering glass tube. By means of the tube the paste can be injected into deep wounds or fistulas.

#### *Technique for the Application of the Chloramine Paste.*

The chloramine paste is designed to maintain in an aseptic condition wounds which have already been disinfected, or to sterilize slightly infected wounds. It should only be applied to wounds which yield small quantities of secretion, have little or no necrotic tissue, and little or no infection.

Neutral sodium oleate is poured on to the wound and the surrounding skin from a flask with a small opening. The granulations, the epithelial edges, and the skin are gently swabbed with a piece of absorbent cotton attached to a forceps. By this means an excellent

<sup>4</sup> Daufresne, M., *J. Exp. Med.*, 1917, xxvi, 91.

cleansing process is effected. The patient should feel no pain; any suffering indicates either that the sodium oleate is incorrectly prepared or that the cleansing is imperfectly carried out. The sodium oleate is next removed with a plug of cotton soaked in water, and the surface of the skin is dried by carefully applying a compress of absorbent gauze.

A sufficient quantity of chloramine paste is withdrawn from the receptacle by means of a sterilized wooden spatula and applied to the surface of the wound to the thickness of at least 1 cm. It should cover not only the granulations, but also the epithelial edges and part of the surrounding skin. If the wound is deep and anfractuous the tube containing the chloramine paste is introduced into the opening, and sufficient chloramine paste is expressed to fill the cavity. But no pressure should be applied during the process.

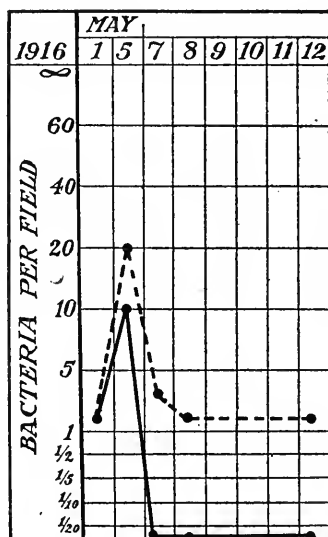
A compress of dry gauze, which should be much larger than the wound itself, is next placed over the chloramine paste. The compress is applied to the surface of the skin and attached to it by means of two or three strips of adhesive plaster. It is important that the gauze should be placed exactly over the wound, for if the bandage is shifted the gauze will introduce bacteria from the surrounding skin on to the surface of the granulations and reinfection will ensue. Above the gauze is placed a piece of absorbent cotton enveloped in gauze. The dressing must not be compressed by bandages and should be renewed every 24 hours. The wound is washed out with sodium oleate every day or two, depending on the condition of the skin. The application of chloramine should be painless; any sensation of pain signifies a technical error on the part of the surgeon.

The bacteriological condition of the wound is examined every day in film preparations of secretions taken from various parts of the wound.

*Effect of a Paste Containing 4 Parts per 1,000 of Chloramine-T upon the Bacteriological Condition of an Aseptic or Slightly Infected Wound.*

The influence of sodium stearate containing 4 parts per 1,000 of chloramine-T was first tested on surface wounds which had been rendered almost aseptic by instillations of Dakin's hypochlorite solution.

In the first experiment a comparison was made of the effect on the bacteriological condition of a slightly infected wound of sodium stearate alone, and of sodium stearate containing 4 parts per 1,000 of chloramine-T.



TEXT-FIG. 1. Experiment 1. Case 366. Comparative action of sodium stearate and of sodium stearate containing 4 parts per 1,000 of chloramine-T. Wound in back of thigh. The upper part of the wound is dressed with sodium stearate containing 4 parts per 1,000 of chloramine-T. The lower part is dressed with sodium stearate. The upper half of the wound is sterile, while the lower half remains unchanged. The solid line indicates the upper part of the wound; the broken line, the lower part.

*Experiment 1. Case 366. Comparison of the Action of Chloramine-T and of Sodium Stearate.*—Elongated wound at the back of the thigh.

May 4, 1916. The area of the wound is 23 sq. cm., and the surface averages 1 bacterium per field (Text-fig. 1). The upper half of the wound is dressed with sodium stearate containing 4 parts per 1,000 of chloramine-T, and the lower half is dressed with sodium stearate.

May 5. The upper half of the wound contains 10 bacteria per field, and the lower half 20 per field. Same dressing.

May 6. Same dressing.

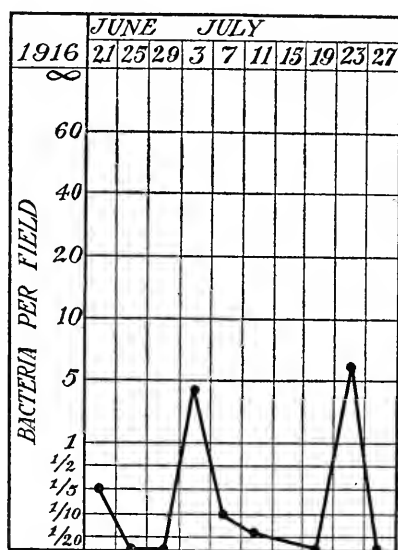
May 7. The upper half of the wound is aseptic; the lower part contains 3 bacteria per field. Same dressing.

May 8. The upper part of the wound is aseptic; the lower half contains 1 bacterium per field. Same dressing.

May 12. The upper half has no bacteria, and the lower half 1 per field.

This observation shows, on the one hand, that sodium stearate has no effect on a slightly infected wound, and, on the other hand, that sodium stearate containing 4 parts per 1,000 of chloramine-T produces surgical asepsis. The bacteria disappeared completely from the films taken from the portions of the wound treated with chloramine-T, whereas they were present in all the films from the part not so treated.

In the following experiments it was attempted to maintain in an aseptic condition wounds which had been rendered surgically sterile at the beginning of treatment.



TEXT-FIG. 2. Experiment 2. Case 488. Preservation of asepsis in a wound by treatment with chloramine-T paste, 4 parts per 1,000. Wound at back of right leg. Two slight reinfections.

*Experiment 2. Case 488. Slight Reinfection of a Wound Treated with Chloramine-T, 4 Parts per 1,000.*—Wound at back of right leg. Treated with sodium hypochlorite solution.

June 17, 1916. The wound contains but 1 bacterium in 5 fields. Dressing with chloramine-T, 4 parts per 1,000.

June 21. 1 bacterium in 5 fields (Text-fig. 2).

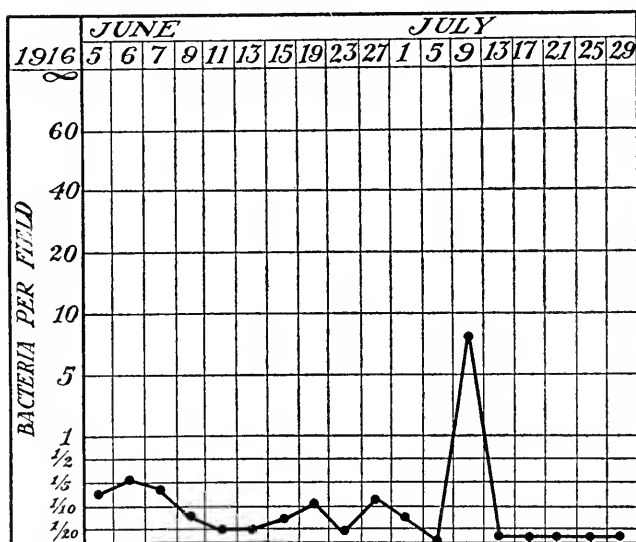
June 25-29. No bacteria.

July 3. Reinfection; 4 bacteria per field.

July 7-19. 1 bacterium in 20 fields; sometimes none.

July 23. Slight reinfection; 6 bacteria per field.

July 27. No bacteria; complete healing.



TEXT-FIG. 3. Experiment 3. Case 491. Effect of chloramine-T, 4 parts per 1,000, upon the asepsis of a wound.

*Experiment 3. Case 491. Wound Kept Aseptic with Chloramine-T, 4 Parts per 1,000. Slight Reinfection.*—Wound on inner side of right thigh.

Apr. 13, 1916. Wound is covered with necrotic tissue. Moderate infection. Sterilization with sodium hypochlorite.

June 3. The wound is surgically sterile.

June 4. Wound is granulating and still shows small sections of necrotic aponeurosis. In these parts alone about 3 bacteria per field are found. The major portion of the wound is covered with surgically sterile granulations. Washing with neutral sodium oleate; dressing with 4 parts per 1,000 of chloramine-T.

June 5-15. The wound is dressed in the same manner and remains surgically sterile (Text fig. 3).

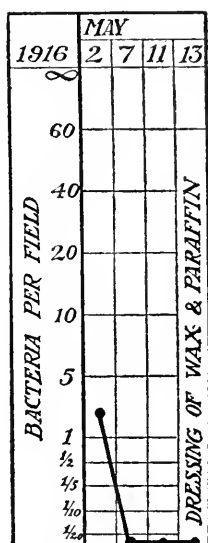
July 9. Reinfection of wound; 8 to 10 bacteria per field. Dressing with chloramine-T, 10 parts per 1,000.



July 13. Wound is sterile.

July 14. Chloramine dressing, 4 parts per 1,000.

July 13-29. Wound has remained sterile.



TEXT-FIG. 4. Experiment 4. Case 450. Effect of chloramine-T, 4 parts per 1,000, upon the asepsis of a wound.

*Experiment 4. Case 450. Sterilization of a Slightly Infected Wound and Preservation of Asepsis with Chloramine-T, 4 Parts per 1,000.*—Wound on outer surface of right leg; sterilized with sodium hypochlorite.

May 2, 1916. The secretions contain 2 bacteria per field (Text-fig. 4).

May 3. Wound is washed with neutral sodium oleate; dressing with chloramine-T, 4 parts per 1,000.

May 7. All the bacteria have disappeared.

May 13. Grafts of skin are applied to the granulations and the wound is dressed with wax and paraffin.

*Experiment 5. Case 327. Reinfection of a Sterile Wound Treated with Chloramine-T, 4 Parts per 1,000.*—Wound in the antero-external region of the left thigh; sterilized with sodium hypochlorite and chloramine-T solution.

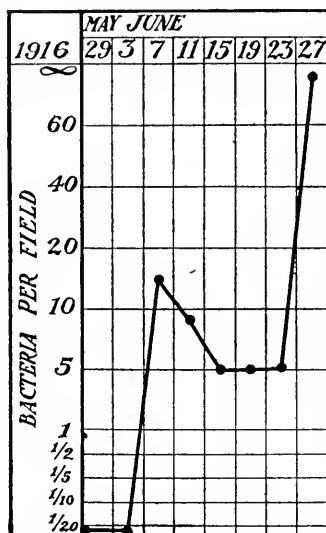
May 25, 1916. The wound, which is 25 sq. cm. in area, is surgically sterile. Dressing with chloramine-T, 4 parts per 1,000.

May 29. Wound sterile (Text-fig. 5); same dressing.

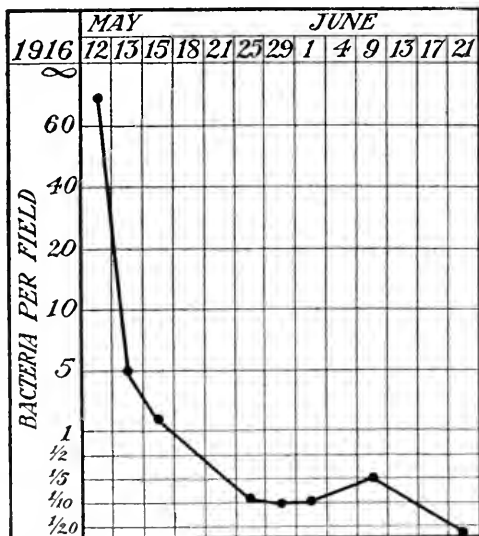
June 3. Wound sterile; same dressing.

June 7. Reinfection; same dressing.

June 27. Large number of bacteria.



TEXT-FIG. 5. Experiment 5. Case 327. Reinfection of an aseptic wound in spite of treatment with chloramine-T, 4 parts per 1,000.



TEXT-FIG. 6. Experiment 6. Case 445. Infection of cutaneous origin of a smooth and granulous wound in the foot. Sterilization with chloramine-T, 4 parts per 1,000.

*Experiment 6. Case 445. Sterilization with Chloramine-T, 4 Parts per 1,000, of a Small, Slightly Infected Surface Wound.*—Wound in the dorsal region of the foot.

May 12, 1916. Wound shows grayish granulations and the secretions contain approximately 100 bacteria per field (Text-fig. 6). Dressing of chloramine-T, 4 parts per 1,000.

May 29. Wound is surgically sterile and remains sterile through June 21, at which date complete healing is effected.

It has thus been shown that wounds in the fleshy regions could be maintained in a condition of surgical asepsis with sodium stearate containing 4 parts per 1,000 of chloramine-T. It was also possible to sterilize wounds presenting a slight infection of cutaneous origin. But in other cases the 4 parts per 1,000 of chloramine-T failed to maintain the asepsis. In Experiment 5 cutaneous reinfection developed in spite of the daily application of chloramine-T. The same occurred in Experiments 2 and 3, in which bacteria reappeared. It is probable, therefore, that the concentration of chloramine-T was too weak.

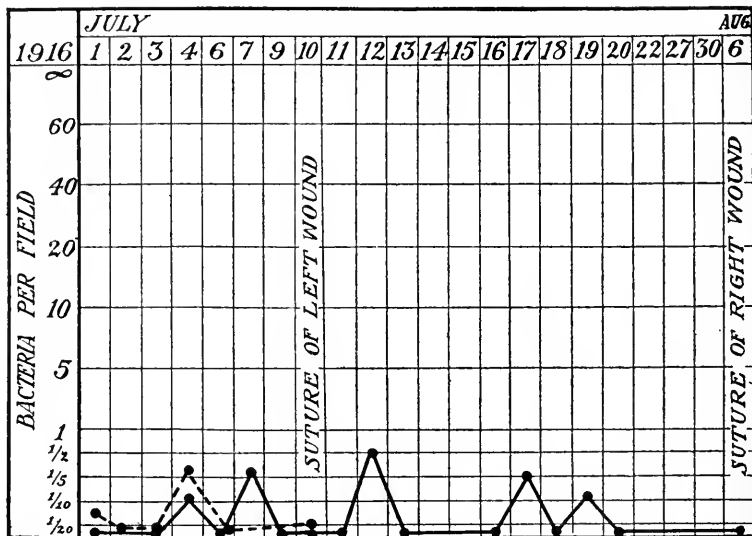
*Effect of a Paste Containing 10 Parts per 1,000 of Chloramine-T upon the Bacteriological Condition of an Aseptic or Slightly Infected Wound.*

Wounds, in some cases accompanied by fracture, were sterilized with Dakin's solution, and then dressed with chloramine-T, 10 parts per 1,000.

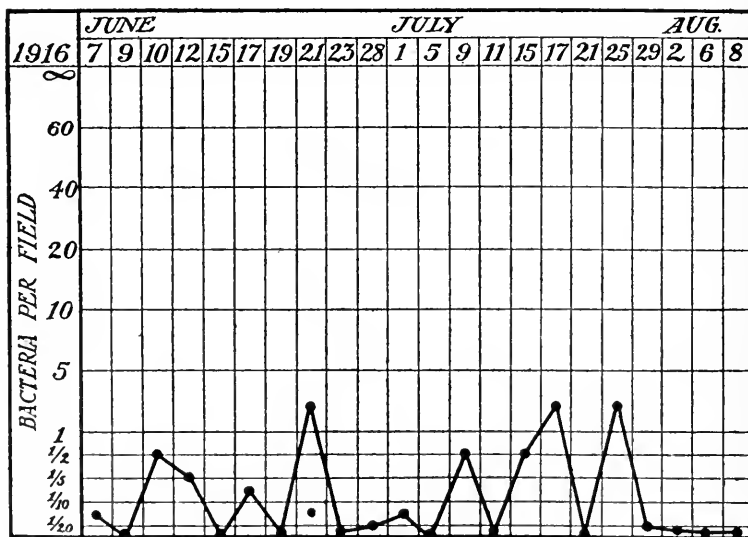
*Experiment 7. Case 548. Preservation of Asepsis with Chloramine-T, 10 Parts per 1,000, of Two Penetrating Wounds in the Region of the Buttocks, Accompanied by Extensive Tissue Detachment.*—Deep wounds in both buttocks caused by a projectile which entered the right thigh, came out between the buttocks in the anal region, and penetrated the left buttock, where it produced extensive muscular detachment. The large wounds and the intramuscular channels were sterilized with Dakin's hypochlorite solution.

July 1, 1916. The right wound has a surface area of 4 sq. cm., and the left of 10 sq. cm. These wounds communicate by means of deep anfractuous passages with the wounds located in the vicinity of the anus. Chloramine-T, 10 parts per 1,000, is carefully injected into the channels. This dressing is repeated daily.

July 6. The lower portion of the left wound has closed (Text-fig. 7). The deep channels healed spontaneously; the cavity separating the right wound from the anal region was kept in an aseptic condition by means of chloramine-T, and its sides grew together without the need of counter-incision.



TEXT-FIG. 7. Experiment 7. Case 548. Preservation of asepsis of two deep wounds in the region of the buttocks by the use of chloramine-T, 10 parts per 1,000. The solid line indicates the right wound; the broken line, the left wound.



TEXT-FIG. 8. Experiment 8. Case 516. Preservation of asepsis in an open fracture by means of chloramine paste, 7 and 10 parts per 1,000.

July 10. Closing of the left wound.

Aug. 6. The right wound is sutured. The curve indicated that the wounds remained aseptic during the treatment.

*Experiment 8. Case 516. Maintenance of an Osseous Cavity in an Aseptic Condition with Chloramine-T, 7 and 10 Parts per 1,000.*—May 17, 1916. Fracture of the upper part of the left tibia. Local sterilization with sodium hypochlorite.

June 4. At the level of the fracture there is a cavity the size of a small egg, containing grayish secretions. The opening of the cavity is at the base of a wound situated on the inner surface of the leg. The wound and cavity are surgically sterile. They are filled with chloramine-T paste, 5 parts per 1,000.

June 6. The paste has not been absorbed. Between the paste and the surface of the cavity is a fluid, transparent substance. Slight reinfection; 4 bacteria per field. Chloramine dressing, 7 parts per 1,000.

June 7. Wound is sterile (Text-fig. 8).

June 13. Wound is sterile. After the osseous cavity has been filled with chloramine paste a portion of the wound is sutured, but the loss of cutaneous substance effected at the time the wound occurred prevents its complete closure. The open part of the wound is covered with chloramine paste, 7 parts per 1,000. The wound remains aseptic.

July 10. Chloramine paste is removed from the osseous cavity. The walls of the cavity are seen to be covered with granulations. Without removing the paste which remains in the cavity, the latter is filled with chloramine paste, 10 parts per 1,000. The osseous cavity has remained completely aseptic. The slight reinfections shown by the curve exist only at the edges of the wound.

Aug. 22. The cavity has almost filled and is covered with epithelium.

*Experiment 9. Case 625. Preservation of Asepsis in Five Flesh Wounds with Chloramine-T, 10 Parts per 1,000.*—Numerous wounds in the left leg produced by bursting shells.

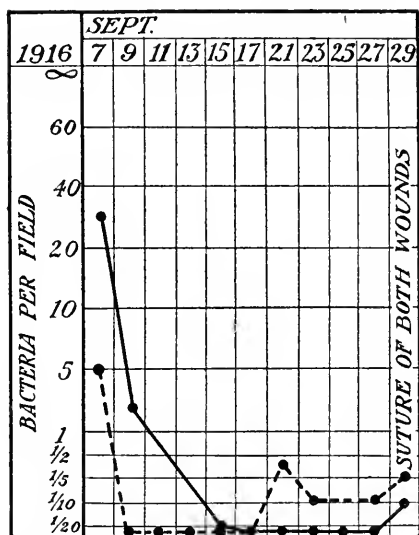
Sept. 5, 1916. Patient admitted to the hospital. The wounds are covered with pus and are treated with sodium hypochlorite.

Sept. 11. The pus has disappeared and the wounds are sterile (Text-fig. 9). Chloramine dressing, 10 parts per 1,000.

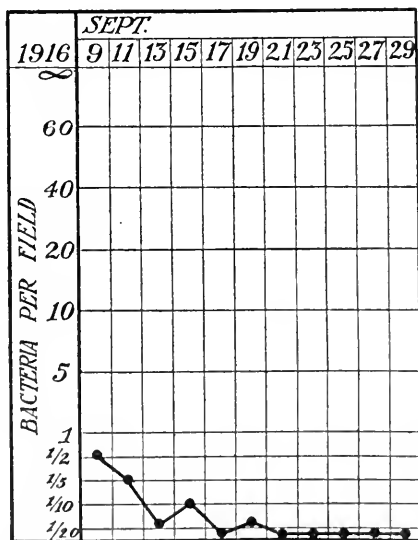
Sept. 13. Suture of three of the wounds; the other two are dressed with chloramine-T. They remain sterile through Sept. 29, when they are sutured.

*Experiment 10. Case 590. Preservation of Asepsis in a Flesh Wound with Chloramine-T, 10 Parts per 1,000.*—Wounds in the right thigh, accompanied by femoral fracture, produced by bursting shells. Treated with sodium hypochlorite.

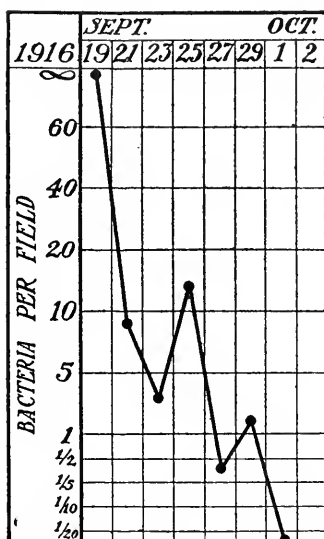
Sept. 11, 1916. The anterior wound is surgically aseptic (Text-fig. 10). Chloramine dressing. The wound is separate from the site of the fracture and remains sterile through Sept. 29 under chloramine dressing.



TEXT-FIG. 9. Experiment 9. Case 625. Preservation of asepsis of flesh wounds with chloramine-T, 10 parts per 1,000. The solid line indicates the posterior wound; the broken line, the anterior wound.



TEXT-FIG. 10. Experiment 10. Case 590. Preservation of sterility in a wound accompanied by femoral fracture with chloramine-T, 10 parts per 1,000.



TEXT-FIG. 11. Experiment 11. Case 620. Sterilization of a slightly infected wound with chloramine-T, 10 parts per 1,000.

*Experiment 11. Case 620. Sterilization of a Slightly Infected Flesh Wound with Chloramine-T, 10 Parts per 1,000.*—Numerous shell wounds. A large wound in the left buttock is treated with sodium hypochlorite. It is gradually cleansed.

Sept. 25, 1916. Wound contains 10 to 20 bacteria per field (Text-fig. 11). There are practically no secretions. Chloramine dressing, 10 parts per 1,000.

Sept. 27. Wound is surgically aseptic; same dressing.

Oct. 1. The bacteria have completely disappeared.

*Experiment 12. Case 634. Reinfection Followed by Sterilization of Two Flesh Wounds with Chloramine-T, 10 Parts per 1,000.*—Numerous shell wounds on the forehead, shoulder, and elbow. Disinfection of wounds with Dakin's hypochlorite solution.

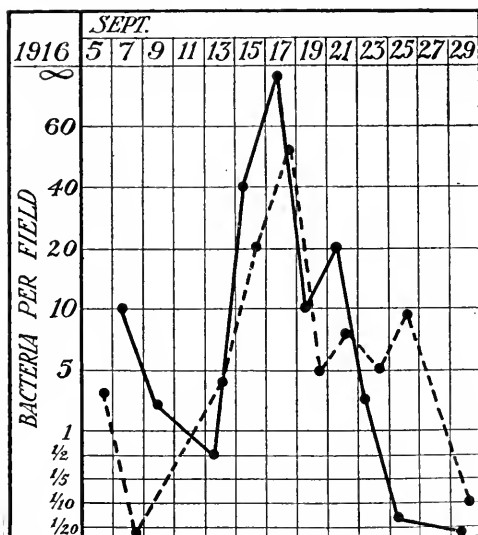
Sept. 11, 1916. The wounds on the forehead and left shoulder are almost sterile (Text-fig. 12). Application of chloramine paste and gauze dressing. The patient, who has also a severe fracture of the elbow and several other wounds, is very restless and the surface dressings become displaced.

Sept. 15 and 17. Reinfection from the skin. Considerable increase in the number of bacteria.

Sept. 17. Washing with neutral sodium oleate. The chloramine dressing is firmly fixed in place.

Sept. 19 and 21. Rapid diminution in the number of bacteria. Same dressing.

Sept. 29. Both wounds are sterile.



TEXT-FIG. 12. Experiment 12. Case 634. Reinfection of cutaneous origin, caused by shifting of dressing, and subsequent sterilization with 10 parts per 1,000 of chloramine-T. The solid line indicates the wound on the forehead; the broken line, the wound in the left shoulder.

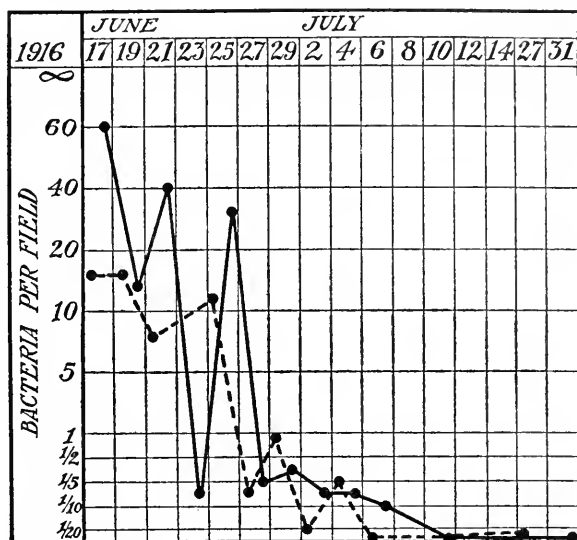
The six experiments given above were selected from a series of sterile or slightly infected wounds treated with chloramine-T paste, 10 parts per 1,000. In every case the chloramine-T either maintained or produced the sterility of wounds infected from the skin. Nevertheless, this result can only be obtained if the dressings are made according to the technique described above. In Experiment 12, the dressings had not been firmly fixed at the surface of the wounds. The gauze shifted slightly and a cutaneous reinfection occurred. But sterilization was reestablished as soon as the appropriate technique was applied.

*Sterilization of a Wound with Chloramine-T, 10 Parts per 1,000.*

The above experiments and many others having shown that chloramine-T, 10 parts per 1,000, preserves a wound in an aseptic condition for several days and weeks, we next endeavored to ascertain whether it would also effect the sterilization of an infected wound.



The first of these experiments was made with surface wounds showing no necrotic tissue and slight infection. Later they were applied to wounds accompanied by a fracture.



TEXT-FIG. 13. Experiment 13. Case 519. Sterilization of two surface wounds of the left thigh with chloramine-T, 10 parts per 1,000. The solid line indicates the inside wound; the broken line, the posterior wound.

*Experiment 13. Case 519. Sterilization of Two Flesh Wounds with Chloramine-T, 10 Parts per 1,000.*—Two wounds of the thigh sterilized with sodium hypochlorite; subsequent reinfection. The wound on the inner side measures 24 sq. cm.; that towards the back, 19 sq. cm.

June 25, 1916. Both wounds are covered with smooth red granulations. No necrotic tissue; small amount of secretion; slight infection. Cocci, diplococci, and bacilli appear on the inside wound in the proportion of 30 per field, and 12 per field in the posterior wound (Text-fig. 13). Application of chloramine paste, 7 parts per 1,000.

June 26. Inside wound shows cocci, diplococci, and streptococci averaging 10 to 15 per field. In the posterior wound there are masses of cocci—6 to 8 per field. Washing with neutral sodium oleate; dressing with chloramine paste, 7 parts per 1,000.

June 27. Inside wound contains 1 coccus in 5 fields; posterior wound contains 1 coccus in every 7 fields.

June 30. Chloramine dressing, 10 parts per 1,000.

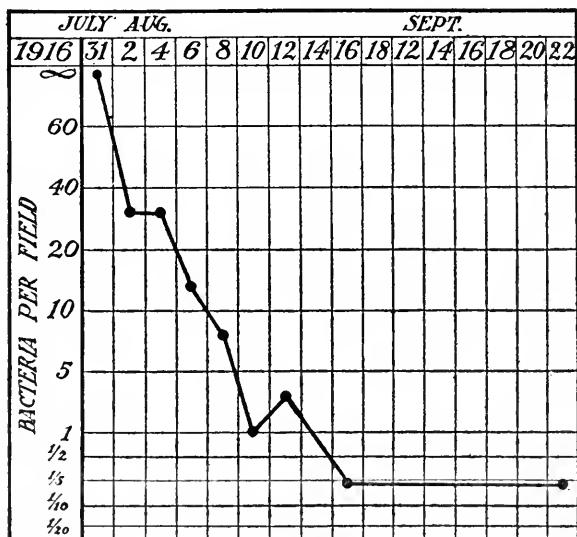
July 1. Inner wound shows 1 coccus in 8 fields; posterior wound shows 1 coccus in 8 fields. Same dressing.

July 4. Few bacteria. From this time on the wounds remain sterile.

July 27. The posterior wound has healed.

July 31. The inside wound has healed.

This experiment shows that a wound covered with smooth granulations, devoid of necrotic tissue, with a slight quantity of secretion, and a varied but sparse bacterial flora, may be completely sterilized with chloramine-T paste. The experiment was repeated with a large number of wounds and always produced identical results. Thereafter the same treatment was applied to more heavily infected wounds which still contained some necrotic tissue.



TEXT-FIG. 14. Experiment 14. Case 591. Sterilization of a seton wound in the sole of the foot with chloramine-T, 10 parts per 1,000.

*Experiment 14. Case 591. Sterilization of a Seton Wound in the Sole of the Foot with Chloramine-T, 10 Parts per 1,000.*—Seton wound in the sole of the foot produced by a fragment of a torpedo on July 24.

July 31, 1916. The wound is very much inflamed and infected. Large number of bacteria (Text-fig. 14). Treatment with Dakin's hypochlorite solution.

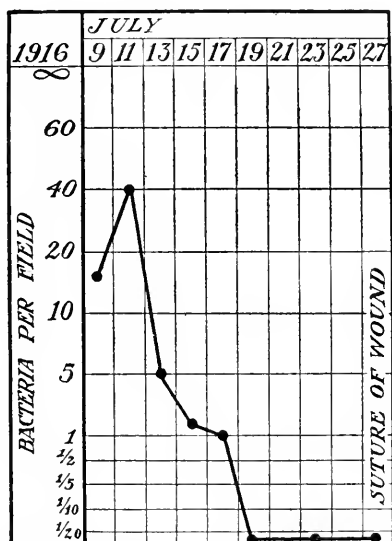
Aug. 3. Sole of the foot still infected. At the surface a small amount of necrotic tissue remains. Radiological examination of the wall of the wound shows the presence of nine minute fragments which cannot be removed owing to their small size. Injection of chloramine paste into the channel and chloramine dressing, 10 parts per 1,000.

Aug. 4. The wound still contains 30 bacteria per field.

Aug. 6 and 8. The wound is becoming sterile.

Aug. 10. The wound now contains only 1 bacterium per field.

Aug. 16. The wound has become surgically sterile. It remains in this condition through Sept. 22, when complete sterilization is effected.



TEXT-FIG. 15. Experiment 15. Case 562. Sterilization with chloramine-T, 10 parts per 1,000, of a slightly infected wound in the right buttock, accompanied by fracture of the ilium.

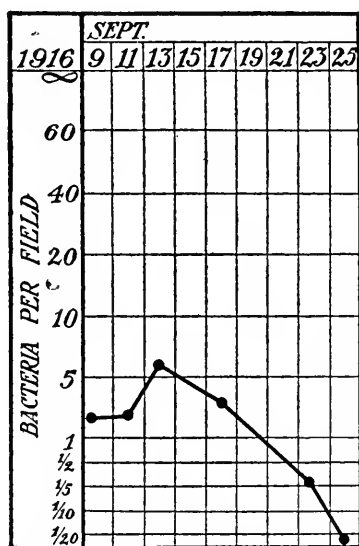
*Experiment 15. Case 562. Sterilization of a Wound Accompanied by Fracture with Chloramine-T, 10 Parts per 1,000.*—July 4, 1916. Large shell wound in the upper part of the right buttock and fracture of the ilium. Wound is first treated with Dakin's hypochlorite solution.

July 9. The surface of the wound is cleansed. The secretions average 15 bacteria per field (Text-fig. 15). Dressing with chloramine paste, 10 parts per 1,000.

July 10. Wound is covered throughout with red granulations; only at isolated points is it still grayish in color. Chloramine dressing, 10 parts per 1,000.

July 13. 5 bacteria per field.

- July 17. The grayish spots have disappeared. About 1 bacterium per field.  
 July 20. Wound is sterile.  
 July 27. Wound is sutured.



TEXT-FIG. 16. Experiment 16. Case 646. Sterilization with 10 parts per 1,000 of chloramine-T of a deep, slightly infected wound.

*Experiment 16. Case 646. Sterilization of a Slightly Infected Flesh Wound with Chloramine-T, 10 Parts per 1,000.*—Sept. 8, 1916. Numerous suppurating and severely infected wounds on the left leg. Two of the wounds are treated for 48 hours with Dakin's hypochlorite solution.

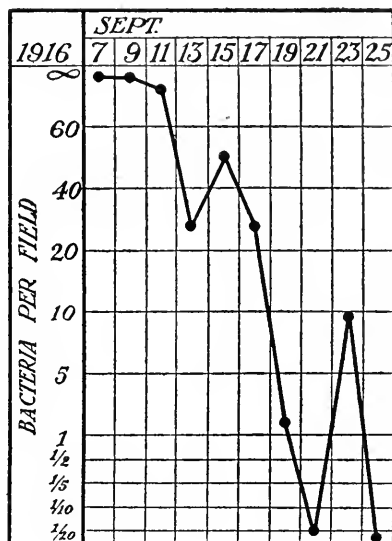
Sept. 11. The inside wound is still infected (Text-fig. 16). It is deep, and the calf is still inflamed. Chloramine dressing, 10 parts per 1,000.

Sept. 23. Wound is surgically sterile.

*Experiment 17. Case 441. Sterilization of a Small, Severely Infected Wound with Chloramine-T, 10 Parts per 1,000.*—Sept. 4, 1916. Removal by a bursting shell of the fourth and fifth fingers of the left hand, and of the greater part of the fifth metacarpal.

Sept. 6. Suppurating wound, severely infected and painful, containing necrotic tissue and large numbers of bacteria. Treatment for 5 days with Dakin's hypochlorite solution.

Sept. 11. The wound still contains large numbers of bacteria in the necrotic parts (Text-fig. 17). Red granulations have appeared. Dressing with chloramine paste, 10 parts per 1,000.



TEXT-FIG. 17. Experiment 17. Case 441. Sterilization of a severely infected wound containing a small quantity of necrotic tissue with chloramine-T, 10 parts per 1,000.

Sept. 13. The wound contains 30 bacteria per field. Same dressing.

Sept. 19. Wound is almost sterile; contains 1 bacterium per field.

Sept. 21. A few bacteria are found on a small fragment of necrotic aponeurosis.

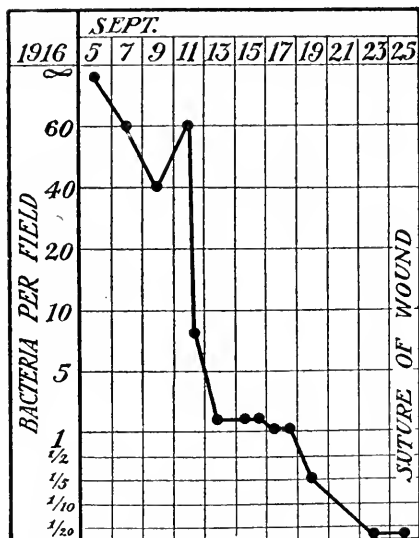
Sept. 25. Wound is sterile.

*Experiment 18. Case 626. Sterilization with Chloramine-T, 10 and 15 Parts per 1,000, of a Large, Severely Infected Wound, Accompanied by Fracture.*—Extensive wound, more than half the thickness of the forearm in depth, with fracture of the radius and cubitus.

Sept. 5, 1916. The wound, which is 9 days old, appears grayish in color. The muscular and osseous surfaces have been evened, but they are still covered with necrotic tissue and blue pus. The limb is swollen and painful. Chloramine dressing, 10 parts per 1,000.

Sept. 7. The blue pus has almost completely disappeared. At the surface and bottom of the wound some necrotic tissue remains. Application of chloramine paste, 15 parts per 1,000, at the level of the osseous extremities. The rest of the wound is covered with chloramine-T, 10 parts per 1,000.

Sept. 13. The wound now contains 1 bacterium per field (Text-fig. 18). The granulations of the section of the wound treated with chloramine paste, 15 parts per 1,000, have assumed a deep red color. The necrotic tissue has completely disappeared.



TEXT-FIG. 18. Experiment 18. Case 626. Sterilization with 10 and 15 parts per 1,000 of chloramine-T of a wide, severely infected wound of the forearm, accompanied by fracture of the upper part of both bones, and with pyocyanic infection and necrotic tissue.

Sept. 19. Wound is surgically sterile.

Sept. 23. The bacteria have disappeared from the films.

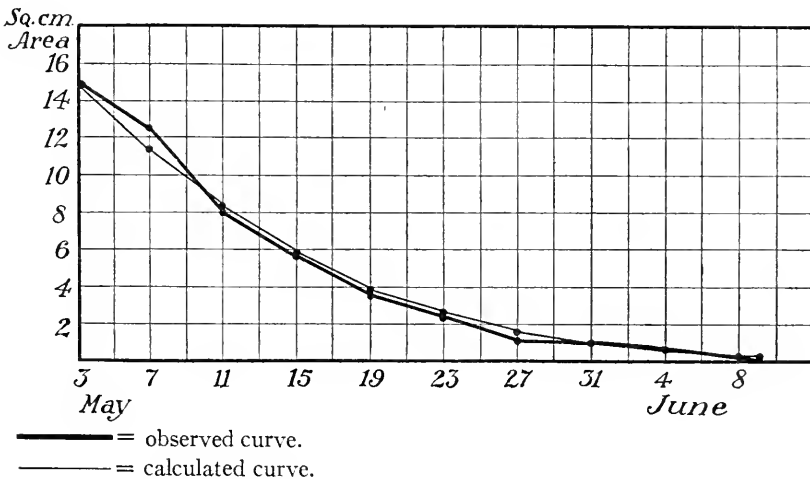
Sept. 25. Wound is sutured.

The wounds in the above experiments were in different stages of infection. Some were simple flesh wounds unaccompanied by necrotic tissue and with but slight suppuration. Others showed necrotic tissue and greater suppuration, while in a third group cases of severe local infection were accompanied by fracture. The wound in Experiment 18 was complicated by fracture of the radius and ulna and showed abundant suppuration. Moreover, it contained a large number of *pyocyanus* bacilli. All these wounds were easily sterilized with chloramine-T paste, 10 or 15 parts per 1,000. In 48 hours the blue pus had almost completely disappeared from Wound 18. But sterilization took place more slowly than would have been the case if instillations of sodium hypochlorite had been used. These results were obtained with a paste containing 10 to 15 parts per 1,000 of

chloramine-T. The 15 parts per 1,000 of chloramine-T caused slight irritation to the skin and congested the granulations. Apparently the concentration of chloramine-T must not exceed 10 parts per 1,000.

*Effect of Chloramine Paste on the Rate of Cicatrization.*

In the following experiments an attempt was made to determine whether chloramine-T, when used in a concentration of 4 and of 10 parts per 1,000, has any effect upon the process of cicatrization. The chloramine paste was applied to wounds whose surface was measured every 4 days, and whose healing curve had been calculated according to the formula of du Noüy. By comparing the curves as calculated and observed we were able to determine whether the action of the chloramine paste retarded the process of cicatrization. Experiments were made with surface wounds treated with chloramine-T in a concentration of 4 and 10 parts per 1,000. The results were approximately identical in all cases. Only two typical experiments will be described.



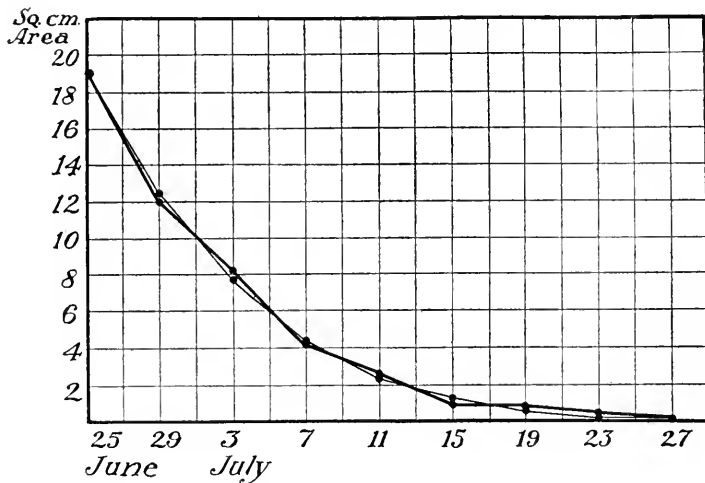
TEXT-FIG. 19. Experiment 19. Case 450. Effect of 4 parts per 1,000 of chloramine upon the rate of cicatrization of a wound. Application of chloramine paste on May 3 to 13.

*Experiment 19. Case 450. Effect of Chloramine-T, 4 Parts per 1,000, on the Cicatrization of an Aseptic Wound.—Granulous wound.*

May 3, 1916. Wound almost sterile and contains only a few bacteria. The surface measures 15 sq. cm. The cicatrization curve, calculated according to the formula of du Noüy, shows that the healing process should be accomplished on June 8. Chloramine dressing, 4 parts per 1,000.

May 7. The wound is sterile; same dressing. From this time on the cicatrization curve descended and caught up with the calculated curve (Text-fig. 19).

From May 11 to 13, at which time wax was substituted for the chloramine-T, the curve followed the calculated curve.



TEXT-FIG. 20. Experiment 20. Case 519. Effect of chloramine-T, 7 and 10 parts per 1,000, upon the rate of cicatrization of a wound. The wound was dressed from June 27 to 30 with chloramine-T, 7 parts per 1,000, and from June 30 to July 27 with chloramine-T, 10 parts per 1,000.

*Experiment 20. Case 519. Effect of Chloramine-T, 7 and 10 Parts per 1,000, upon the Cicatrization of an Aseptic Wound.*—Granulating wound at the back of the thigh.

June 25, 1916. Superficial measurement, 19 sq. cm. The secretions contain 10 bacteria per field. The course of cicatrization is calculated according to the formula of du Noüy.

June 27. The wound is surgically aseptic. Chloramine dressing, 7 parts per 1,000, is used until June 30.

June 30 to July 27. Chloramine dressing, 10 parts per 1,000. The curve coincided almost exactly with the calculated curve; nevertheless, after July 19 slight retardation of the healing process apparently occurred (Text-fig. 20).



The above experiments, as well as others that are analogous, show that sodium stearate containing from 4 to 10 parts per 1,000 of chloramine-T does not retard the process of cicatrization to an appreciable extent. In Experiment 20 during the first 25 days the observed curve coincided with the calculated curve, after which the rate of healing became slightly reduced.

#### SUMMARY.

Sodium stearate has no effect upon the bacteriological condition of a wound, but the addition of 4 parts per 1,000 of chloramine-T renders it antiseptic. Experiment 1 enabled us to compare the action of sodium stearate alone with that of sodium stearate containing 4 parts per 1,000 of chloramine-T. Wounds which had been previously sterilized could be maintained in an aseptic condition by 4 parts per 1,000 of chloramine-T, although in some cases reinfection occurred (Experiments 2, 3, and 5). For this reason the concentration of chloramine-T was increased.

Surface wounds, deep-seated wounds, and osseous cavities, which had previously been either completely or almost completely sterilized, could be maintained for days and even weeks in a condition of surgical asepsis by the use of a paste containing 7 and 10 parts per 1,000 of chloramine-T. Experiments 7, 8, 9, 10, and 12 are examples of this. Slightly infected wounds (Experiments 9, 11, and 12) were sterilized in the same manner.

Next, it was attempted to sterilize wounds which were suppurating and more or less infected, and in some cases accompanied by fracture. This attempt was probably successful because the wounds used for the experiments showed but slight quantities of secretions and only a shallow layer of necrotic tissue. It is useless to attempt to sterilize severely infected wounds with a paste, for the volume of chloramine-T that can be applied is too limited. A large volume of an active substance is required to sterilize a wound which secretes great quantities of pus, for owing, on the one hand, to the dilution of this substance with the secretions, and, on the other, to its combination with the proteins contained in the pus, the concentration of the antiseptic is rapidly diminished. For these reasons it is essential that the antiseptic solu-

tion should be constantly renewed, so that the concentration may be sufficiently strong to effect the destruction of the bacteria. Therefore, the chloramine-T paste cannot sterilize a severely infected wound.

The concentration of the active substance contained in a paste must at the same time be sufficiently weak to be innocuous to the tissues. We have seen that it should not exceed 15 parts per 1,000. Thus, it is evident that if the secretions from the wounds are abundant, the substance could exert its action upon the microorganisms for the space of only a few hours. For this reason the chloramine paste should only be applied under the conditions specified in our experiments; that is, in connection with moderately infected wounds which have been carefully washed with sodium oleate, and possess but slight quantities of secretion. Under these conditions, the chloramine paste effects the complete disappearance of the bacteria and maintains the sterility thus secured for as long a time as may be wished. If the technique followed in the dressing is not exactly as above described, reinfection will occur. If applied in this manner the chloramine paste is not injurious to the tissues, for the cicatrization curves of the wounds thus treated show but slight modification from the calculated curves.

Chloramine paste makes it possible, therefore, to keep wounds sufficiently free from microorganisms so that the effect of substances which are believed to influence cicatrization can be studied.

#### CONCLUSIONS.

1. Under the conditions of our experiments chloramine paste maintains the asepsis of a wound already sterile, and sterilizes an infected wound.
2. Under the same conditions chloramine paste causes no apparent modification of the cicatrization curve of an aseptic wound.

# TOXIN AND ANTITOXIN OF AND PROTECTIVE INOCULATION AGAINST BACILLUS WELCHII.

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This study of the pathogenicity of infection by the group of *Bacillus welchii* has followed from several fortuitous circumstances

## ERRATUM.

On page 119, Vol. XXVI, No. 1, July 1, 1917, line 1, read *pathology* for *pathogenicity*.

believed that a better understanding of the one condition would serve to explain many still obscure points in the other.

It will be of interest in this connection to review briefly certain facts concerning gas bacillus infection in the pigeon, since the condition is one little known to pathologists and bacteriologists. The classical article by Welch and Nuttall<sup>1</sup> on the gas bacillus appeared in 1892. It was followed by a paper on gas bacillus infection in man by Welch and Flexner<sup>2</sup> in 1896. The latter article was incomplete,<sup>3</sup> and the concluding part which was to appear in the next number was never published. The second paper was to deal more particularly with experimental gas bacillus infection in animals—in the guinea

<sup>1</sup> Welch, W. H., and Nuttall, G. H. F., *Bull. Johns Hopkins Hosp.*, 1892, iii, 81.

<sup>2</sup> Welch, W. H., and Flexner, S., *J. Exp. Med.*, 1896, i, 24.

<sup>3</sup> Personal communication from Dr. Flexner.

pig and pigeon particularly. Since the pigeon had proved to be highly subject to infection and to respond with characteristic pathologic reactions, that animal came to be employed by the laboratory staff in the more or less routine study of the gas bacillus. But because of the circumstances stated, no full and sufficient description of the local lesions in the pigeon, which in disorganizing effect are comparable with the destructive lesions sometimes present in man, came to be published until some years later, when, at Dr. Flexner's suggestion, Dr. Herter,<sup>4</sup> then engaged in the study of the *Bacillus welchii* group of bacteria occurring in the alimentary tract, employed this animal for inoculation. The lesions as described by Herter agree closely with those present in our pigeons inoculated with cultures of the bacilli.

#### *Sources and Nature of Cultures.*

The main part of our experiments has been made with five strains of *Bacillus welchii*, of which four were obtained through the kindness of Dr. Simonds. The fifth was isolated by us from a piece of clothing which had long been worn. The history of the Simonds cultures follows. The tests given were made by him.

*Strain 365 a.*—Isolated, Aug. 13, 1916, from scrapings from a bullet wound of the thigh, which showed a moderately severe gaseous gangrene. Its virulence for laboratory animals had not been tested.

*Strain 386 cd.*—Isolated, Sept. 9, 1916, from a fragment of shell with adherent bits of clothing, removed from a wound of the thigh. The patient did not develop gaseous gangrene. The organism had not been pathogenic for guinea pigs.

*Strain 617 d.*—Isolated, Aug. 27, 1916, from a case of violent gaseous gangrene following a bullet wound of the thigh with injury to and subsequent ligation of the large vessels of the leg. The limb was amputated. Although the stump was gaseous, the patient recovered. This strain is very pathogenic for guinea pigs, producing typical lesions and killing the animal in less than 24 hours after subcutaneous injection of 0.5 cc. of a 24 hour dextrose broth culture.

*Strain 669 b.*—Isolated, Aug. 21, 1916, from a case of gaseous gangrene following a bullet wound which caused shattering of the lower end of the femur. The leg was amputated; the patient recovered. Injection of 0.5 cc. of a 24 hour dextrose broth culture subcutaneously into a guinea pig was followed by a local-

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<sup>4</sup> Herter, C. A., Bacterial infections of the digestive tract, New York, 1907, 198.

ized gaseous gangrene and sloughing of the skin and subcutaneous tissues. The animal died 2 weeks after the injection. No gas bacilli were found at autopsy in films from tissues adjacent to the slough.

The history of our own strain is as follows: A piece of cloth from the lining of an old overcoat was thrust with tissue forceps deep into the right breast muscle of an anesthetized pigeon. The next morning the wounded muscle was greatly swollen, and crepitation was present. Pressure near the wound forced out gas bubbles which ignited with a snap. The pigeon was drooping and died at 2.30 p.m.

*Autopsy.*—The skin over the right side was edematous and covered with blebs. There was a reddish brown gelatinous exudate in the subcutaneous tissues of both groins and extending over the right pectoral muscles. A film preparation made from this exudate contained a few plump Gram-positive bacilli. The inoculated muscle was edematous and necrotic, most pronounced about the cloth, but extending along the muscle sheaths to the insertion of the fibers. Films from the necrotic muscle contained many plump Gram-positive bacilli and a few Gram-positive diplococci and Gram-negative bacilli.

Tubes of recently boiled litmus milk, blood agar, and blood bouillon were inoculated with the subcutaneous exudate and necrotic muscle. After 24 hours' incubation, the milk tubes showed the so called "stormy fermentation," and those inoculated with the subcutaneous exudate contained a plump Gram-positive bacillus in pure culture. The tubes from the necrotic muscle contained a similar bacillus, together with Gram-positive cocci in pairs and short chains and a Gram-negative bacillus. The blood media inoculated with the subcutaneous exudate remained sterile, while those of the necrotic muscle yielded cocci and Gram-negative bacilli only, the latter proving to be a variety of *Bacillus coli*. We shall call the culture of the Gram-positive bacillus, to be described more fully later, P-50.

The five cultures of Gram-positive bacilli enumerated have been tested for motility, spore formation, quantitative acid and gas production, liquefying action on gelatin at 22° and 37°C., pathogenicity for guinea pigs, rabbits, and pigeons, and still other properties. For example, the several strains have been tested for agglutination and lysis in normal rabbit and guinea pig sera, for hemolysins, for gas production in rabbits after the method of Welch and Nuttall, and for agglutination in artificially produced immune sera. As far as these tests are indicative, they place all five cultures among the group of *Bacillus welchii*; so far as they relate to specific properties, e.g.,

specific and cross agglutination, they indicate certain differences among them such as have commonly been observed among members of the group.

*Pathologic Effect in Animals.*

The feature of most importance among the properties just given which define this group of bacilli is pathogenic effect. This is true in the first place because of the similarity of certain of the lesions in animals to those arising in gaseous gangrene in man; and next and especially, because the investigation of the manner in which the lesions arise in animals led us to the discovery of the conditions under which a highly potent soluble toxic agent is regularly produced by the bacilli on which their poisonous or lethal action chiefly if not wholly depends.

In the past, the laboratory animal usually employed to test pathogenicity of *Bacillus welchii* has been the guinea pig. Many more cultures produce mild local infections, from which recovery takes place, than severer ones which are fatal. But the subcutaneous or intramuscular injection of cultures gives rise to local swelling, gas production, liquefactive necrosis of the involved muscle and skin, and, if the animal survives, eventual sloughing and cicatrization.

The rabbit is more resistant to local infection than is the guinea pig, but nevertheless lesions can be produced by active cultures which in general resemble those of the guinea pig. The rabbit reacts either to more highly virulent cultures or to larger doses of less effective ones. In the case of the former, death may result. But in both the rabbit and the guinea pig general blood invasion, even when the course of the infection is lethal, either does not take place at all or so few bacilli enter the blood that the cause of death cannot be attributed to a septicemia.

The peculiar susceptibility of the pigeon to infection with *Bacillus welchii* has not been utilized to the degree deserved. For in no other laboratory animal is infection produced so readily and with such a wide number of cultures, and in no other does the pathologic process proceed so swiftly and characteristically and with effects so nearly resembling the condition of gaseous gangrene in man. Probably this lack of use of the pigeon is to be ascribed to the rather brief refer-

ences made to the subject in the literature, which is almost confined to a few summary statements emanating from the Johns Hopkins Hospital at about the period already referred to. Dr. Flexner himself employed mainly blood containing large numbers of the bacilli for inoculating pigeons. In some instances the blood was taken post mortem from human cases, and in others from rabbits injected, killed, and incubated by the Welch-Nuttall method. Inoculation from pigeon to pigeon was also carried out. The animals succumbed at periods ranging from 5 to 24 hours. The autopsies revealed lesions precisely like those already described (page 121), except that the injected blood would often remain to tinge the tissues. The bacilli were very numerous in the disorganized muscle, fewer in the gelatinous exudate, and very few in or absent from the heart's blood.

The five cultures were pathogenic for pigeons, although not equally so. Simonds' Culture 617 d proved most virulent, not only for pigeons, but for rabbits and guinea pigs also. Doses of 0.01 to 0.05 cc. of a 20 hour glucose broth culture injected directly into the breast muscles were invariably fatal to pigeons in 6 to 20 hours. 0.0005 cc. of such a fluid culture put upon a bit of gauze and introduced into the muscles usually but not invariably produced fatal infection. Of Cultures 365 a and 669 b approximately 0.1 cc. of a glucose broth culture was the minimal lethal dose; while the dose of Cultures 386 cd and P-50 required to produce corresponding effects was 0.2 to 0.3 cc. The minimal lethal doses for guinea pigs and rabbits were several times those used for the pigeons.

#### *Cause of Death in Bacillus welchii Infection.*

In man, infection with *Bacillus welchii* tends to be a local process, even when severe, and invasion of the general blood occurs if at all only during the death agony or post mortem. In a small number of instances in man general infection seems to have played an important part in causing or hastening death.<sup>5</sup> But as these cases are the exception, even when death occurs, in man as well as in the pigeon, rabbit, and guinea pig, it may be assumed that soluble chemical substances entering the circulation from the local lesion bring about

<sup>5</sup> Thaon, P., *Compt. rend. Soc. biol.*, 1908, lxiv, 863.

the severe symptoms and the fatal termination. The question at once arising from this general consideration relates to the probable nature of the poisonous bodies. Several possibilities present themselves: They may arise from the bacilli, they may be yielded by the disorganizing tissues, or they may be of the nature of acids which disturb profoundly the hydrogen ion concentration of the body fluids. The protocols which follow are given to show the manner in which death may be produced in rabbits and pigeons under circumstances in which the blood remains wholly or practically free of the bacilli.

*Experiment 1.*—Pigeon. 11 a.m. 0.2 cc. of a glucose broth culture of Culture 617 d was injected into the breast muscle. 6.30 p.m. Animal died. Immediate autopsy. Usual gaseous, gelatinous, and necrotic local lesions containing myriads of bacilli. Films from the heart's blood and peritoneal fluid showed no bacilli, although a few were present, as a glucose broth culture was positive.

*Experiment 2.*—Pigeon. 11 a.m. 2 cc. of broth culture of Culture 617 d injected into breast muscles. 2.30 p.m. Animal died, having survived  $3\frac{1}{2}$  hours. Local lesions similar to the preceding experiment, but the films from heart's blood contained a few bacilli.

These experiments were varied and repeated with different doses of the culture, but in no instance did a severe septicemia develop.

*Experiment 3.*—Rabbit. 11 a.m. Sedimented bacilli from 10 cc. of a 24 hour glucose broth culture of Culture 365 a were injected into the ear vein. 12 m. Films from the heart's blood showed a few bacilli, and the culture was positive. 9.30 p.m. Animal stuporous and breathing heavily. 10 p.m. Films and cultures from heart's blood negative. 10.20 p.m. Died. Immediate autopsy showed a dark and large spleen, but blood intact and serum clear. Films and cultures yielded no bacilli from the blood but some from the spleen and liver.

*Experiment 4.*—Rabbit. Mar. 14, 1917. 11 a.m. Sedimented bacilli from 2 cc. of broth culture No. 617 d given intravenously. Mar. 19, 8 a.m. Dead. Autopsy showed bloody fluid in peritoneum and small amount of clear fluid in pleura. The former contained many, the latter few bacilli; the heart's blood contained none.

It is apparent from these experiments that the death of the animals—pigeons and rabbits—is not closely bound up with the multiplication of the bacilli in the general circulation. The rabbit experiments indicate that the bacilli have no power to remain in the blood stream, and even when diminishing in numbers in the internal



organs still exert poisonous effects. Hence it may be concluded that poisons are liberated from the bacilli, but whether merely secreted or only yielded upon disintegration is not indicated by these tests. The next step therefore was to look for soluble toxic substances in the fluid cultures. For this purpose 24 hour glucose broth growths were employed.

*Experiment 5.*—(a) Rabbit; weight 1,950 gm. 8 cc. of Culture 669 b were injected intravenously. Immediately following the injection the animal became greatly excited, respiration became rapid, prostration followed, and death occurred in 7 minutes from respiratory failure.

(b) Rabbit; weight 1,700 gm. 9 cc. of Culture 617 d similarly injected. 14 minutes later this animal suddenly became excited, jumped from the basket, and died 7 minutes later of respiratory failure.

(c) Rabbit; weight 1,800 gm. 10 cc. of Culture 365 a intravenously injected. Died within 6 minutes under similar circumstances to those in (a) and (b).

In other words, an acutely fatal effect can be produced from large quantities of a broth culture injected intravenously. Less quantities (1 to 2 cc. per kilo) produce no immediate symptoms but cause death in from 6 to 24 hours. Differences in virulence only appear from the smaller doses and at once distinguish Culture 617 d as the most active. The massive doses of cultures exerted an injurious effect on the red blood corpuscles, which are destroyed in large numbers. On the other hand, the bacilli do not tend to agglutinate rapidly in the blood stream. By performing *intra vitam* agglutination tests, it was found that Culture 386 cd alone became rapidly agglutinated. This one was the least active, requiring the largest dose to cause acute death. The blood became free of the other cultures only after 4 to 8 hours. It appears then that bacillary embolism is not the probable cause of the acute lethal effects.

The toxicity of the fluid portion of the broth cultures was investigated. Since in these tests centrifugation alone was employed, pigeons could not be used, as the small number of bacilli remaining was sufficient to cause infection; hence rabbits were again injected. We may mention here that the injection of 2 to 4 cc. of the centrifuged fluid subcutaneously or intramuscularly into rabbits would sometimes lead to severe local and fatal infection. The fluid therefore possessed aggressive activity.

*Experiment 6.*—(a) Rabbit; weight 1,475 gm. Injected 10 cc. of supernatant fluid of glucose broth culture No. 617 d intravenously. Immediate collapse, air hunger, and death in 2 minutes. The red corpuscles were largely disorganized. The serum was reddish brown, and the remaining corpuscles appeared as mere shadows.

(b) Rabbit; weight 1,400 gm. Before injection the red corpuscles numbered 5,600,000 and the white 8,700 per c.mm. 12 m. 4 cc. of supernatant fluid of Culture 617 d were injected intravenously. 20 minutes later breathing was rapid and labored; the animal was very weak, and the red cells had fallen to 80,000 per c.mm. The white cells were unchanged. 1.20 p.m. Animal prostrate, the red cell count 84,000, and the white cells 8,300 per c.mm. 10 minutes later respiration ceased. The kidneys were dark brown in color, the bladder contained dark brown urine, and the few remaining red corpuscles were shadowy.

(c) Rabbit; weight 1,375 gm. Red cells 5,250,000 per c.mm. 11.50 a.m. Received 3 cc. of supernatant fluid, Culture 617 d. 12.15 p.m. Respiration accelerated, red cells 1,600,000. 3 p.m. Red cells 1,650,000. Died in night. The urine found was dark brown in color. Kidneys chocolate colored.

It would appear to be shown by these experiments that the acutely fatal effects of massive doses of the broth cultures as such or when separated in large part from the bacilli themselves are due to some body causing rapid and extensive blood destruction. Whether any other factor plays a part these experiments do not determine.

The next experiments were devised to rule out the factor of acidity. The different supernatant fluids exhibited acidities ranging from 2.5 to 4.5 per cent in terms of normal sodium hydroxide, with phenolphthalein as indicator. References in the literature point to the acid and especially the butyric acid content of cultures as responsible for the toxic effects. Two sets of tests were made (1) by neutralizing the broth with sodium hydroxide and (2) by comparing the acidity with the toxicity of different fluids.

*Experiment 7.*—Rabbit; weight 1,575 gm. Red cells 5,450,000. 12 m. 5 cc. of supernatant fluid of Culture 617 d neutralized with sodium hydroxide were injected intravenously. 12.35 p.m. Red cells 1,600,000. 1.50 p.m. Red cells 800,000. Respiration rapid, animal tires easily. 3 p.m. Red cells 600,000. Animal died during night. The autopsy showed the dark urine and chocolate colored kidneys as described above.

The exclusion of the acidity in the fluid may diminish somewhat the intensity of the blood destruction but does not remove it or

prevent the fatal issue. Experiments similar to this were made a number of times with consistent results. Moreover, comparison of toxic action and the degree of acidity was made, from which it was seen that acidity and lethal effects do not proceed hand in hand.

*Experiment 8.*—(a) Rabbit; weight 1,400 gm. Red cells 5,700,000 per c.mm. 2.45 p.m. 8 cc. of supernatant fluid of Culture 617 d, having an acidity of 1.9 per cent normal sodium hydroxide, were given intravenously. 4.45 p.m. Red cells 1,240,000. 5 p.m. Animal breathing rapidly; very weak. Died during night. Usual autopsy findings.

(b) Rabbit; weight 1,600 gm. 2 p.m. 10 cc. of supernatant fluid of Culture 386 cd, having an acidity of 4.5 per cent normal alkali, were injected intravenously. No symptoms appeared, and the red corpuscles were only slightly reduced in number. The animal still lives.

These experiments show that the acidity is not the main factor in causing either blood destruction or the fatal effects, and they are supported by tests on pigeons, examples of which follow; they indicate also that *Bacillus welchii* produces, in the test-tube at least, an active hemolysin.

*Experiment 9.*—(a) Pigeon. Red cells 4,645,000 per c.mm. 9.50 a.m. 0.5 cc. of 24 hour glucose broth culture No. 365 a injected into wing vein. 10.40 a.m. Red cells 4,700,000; no free nuclei present. 11.50 a.m. Pigeon drooping. Red cells 3,880,000, free nuclei appearing. 2 p.m. Red cells 2,432,000; many free nuclei. 3.45 p.m. Dying. Red cells 1,520,000. Immediate autopsy showed some but not numerous bacilli in blood; cytoplasm of the red cells stains weakly; blood serum reddish brown in color.

(b) Similar to (a). In 8 hours, when death occurred, the red cells had fallen from about 5,000,000 per c.mm. to 1,000,000. The autopsy findings were typical.

In other words, the injection of 0.5 cc. of a broth culture of active *Bacillus welchii* intravenously into pigeons causes extensive blood destruction and death in periods of from 6 to 8 hours. These results are now to be contrasted with pigeons in which the culture is injected into the pectoral muscles.

*Experiment 10.*—(a) Pigeon. Red cells, 4,500,000 per c.mm. 9.30 a.m. 0.5 cc. of glucose broth culture No. 365 a injected into breast muscle. 12 m. Drooping. 3.30 p.m. Red cells 4,600,000; no free nuclei. 4.45 p.m. Dying. Red cells 4,450,000. Autopsy showed local infection; no bacilli in blood.

(b) Similar to (a) except that Culture 617 d was employed. At the outset the red cell count was 4,450,000 per c.mm. 6 hours later, when the animal was dying, it was 4,435,000. The autopsy findings were characteristic.

These experiments several times repeated were always consistent. Intravenous injections of broth cultures are attended by extensive blood destruction and death; intramuscular injections of like doses cause death with equal certainty and rapidity but no blood destruction. Hence the blood destruction cannot be the determining factor of the lethal action. The essential toxic agent appears now not to be an acid and not an hemolysin. The next experiments relate to its filterability.

Up to the present the fluid cultures described were not wholly free from the bacilli. To remove the bacilli entirely, in order to test the toxicity of the sterile fluid, filtration through a Berkefeld N candle was resorted to. The first tests were made with filtrates obtained from ordinary glucose broth cultures. They indicated merely a low degree of toxicity for rabbits and pigeons.

*Experiment 11.*—A 24 hour glucose broth growth of Culture 617 d was employed as follows:

(a) Rabbit; weight 1,475 gm. 10 cc. of the supernatant fluid obtained by centrifugation for 20 minutes, not quite clear, and having an acidity of 4.2 per cent were injected intravenously. The animal had a severe convulsion and died almost immediately. The blood was extensively destroyed.

(b) Rabbit; weight 1,425 gm. 11 cc. of a Berkefeld filtrate, having an acidity of 3.9 per cent, were injected intravenously. No symptoms.

(c) Rabbit; weight 1,575 gm. 3 p.m. 10 cc. of the clear fluid obtained by centrifugation for 40 minutes and having an acidity of 4.5 per cent were injected intravenously. For an hour there was respiratory distress, which passed off. 7 p.m. Died. Kidneys chocolate colored. Another part of this fluid first filtered, then injected into a normal rabbit, produced no effect.

This experiment shows that not only does filtration reduce the toxicity, but long centrifugation does also. The difference is not caused by the reduction in acidity observed in the filtered fluid, since neutralization of the centrifugate with sodium hydroxide did not affect its activity. The injection of the filtrate in amounts of 8 cc. into the breast muscles of pigeons caused temporary drooping but no local lesion or other severe effect. No distinction in action was

noted in the different cultures. The conclusion to be drawn from these experiments is that a certain kind of toxic product is developed in glucose broth cultures, but that prolonged centrifugation and filtration tend to remove it from the fluid. Since the action is so rapid, it does not seem probable that there is any actual relationship between the bacilli as such still remaining in the centrifuged fluid and the poisonous agent. The latter is not an ordinary acid and appears to be an hemolysin.

The next experiments throw an entirely different light on the toxin-producing property of *Bacillus welchii*. It is obvious that cultures in glucose broth in no way represent the conditions occurring during local infections in man and animals. Hence these were simulated in the following manner.

To plain beef infusion broth in 10 cc. quantities in test-tubes were added several fragments of sterile skeletal muscle of the pigeon or rabbit. The tubes, having been proved sterile, were inoculated with *Bacillus welchii* and overlaid with paraffin oil and enclosed in a vacuum jar from which the oxygen was exhausted. After an incubation of from 18 to 24 hours, the fluid was centrifuged and filtered through a Berkefeld N candle. The filtrate was always free of the bacilli. This product proved highly toxic for pigeons, guinea pigs, and rabbits, and, what should be emphasized, gave rise to inflammatory and other local lesions resembling closely those caused by the bacilli themselves. While Culture 617 d, the most virulent of all, yielded the most active filtrate, yet all five cultures gave toxic products. The degree and manner of the action of the toxic filtrates are indicated by the following illustrative protocols.

*Experiment 12.*—(a) Guinea pig. 3 p.m. 2 cc. of the Berkefeld filtrate of an 18 hour pigeon muscle broth culture of Culture 617 d were injected beneath the skin of the right thigh. The next morning at 8 a.m., the entire leg was swollen and the joints held stiffly; the scrotum was also edematous. The animal crouched in the corner of the cage. 3 days later the hair was loose, and the tissues were sloughing. Death occurred during the night. The autopsy showed disorganization of the muscles of the right leg and adjacent abdominal wall. Cocci, but no gas bacilli were present.

Doubtless the filtrate acted upon the skin and underlying muscles, inducing inflammation and necrosis, after which pyogenic cocci and

other bacteria entered the injured tissues and produced the sloughing. A dose of 1 cc. of the filtrate caused a similar but less severe lesion, from which the guinea pig slowly recovered after healing of the defect.

(b) Rabbit. 2 cc. of the same lot of toxin used in (a) were injected under the skin of the right thigh at 4.30 p.m. The next day the skin over the leg and the right scrotal sac was highly edematous. 5 days later, the edema subsided, leaving a dry necrotic area behind, which finally was thrown off and became healed.

The local effect, therefore, in the rabbit is similar to although less severe than that in the guinea pig. The effect in the rabbit of an intravenous injection of the same lot of toxin is to produce acute blood destruction and death. Thus a rabbit having a red cell count of 5,400,000 was given 1 cc. of the toxin at 10 a.m. At 11 a.m. the cells numbered 4,250,000; at 12.30 p.m., 2,550,000; at 4 p.m., 1,500,000; at 5 p.m., 1,000,000. Death took place during the night. The kidneys were chocolate colored and the urine dark.

(c) Pigeon. 12 m. 0.2 cc. of a similar toxin was injected into the right breast muscles. The next morning at 8 o'clock there was widespread edema of the injected site. 24 hours later the edema was subsiding. 3 days later the swelling had disappeared. On etherization and autopsy, an extensive necrotic focus was found in the injected pectoral muscles; the muscles of the opposite side were normal.

The local reaction of the pigeon to small doses of the toxic filtrate resembles that of the guinea pig to far larger doses. When 0.3 cc. of the filtrate was injected, the edema developed very quickly and death occurred in about 4 hours—even more quickly, therefore, than from massive bacillary infection. The injected muscle was already friable. No effect is produced in the red blood corpuscles. When, however, the filtrate is injected into the wing vein, hemolysis results. Thus a pigeon having a blood count of 4,280,000 was given 0.25 cc. of neutral filtrate at 9.30 a.m. At 10.30 a.m., the cells were unchanged in number and no free nuclei occurred. At 4.30 p.m. the cells numbered 3,725,000, and free nuclei were found. The next day at 1 p.m. the cells numbered 800,000, and there was marked air hunger. Death took place at 5 p.m.

This experiment, which was repeated several times, shows that *Bacillus welchii*, when the conditions of growth are suitable, yields toxic products of high potency. These products produce two sets of effects according to the manner of their injection into animals: (a) hemolysis, in which they resemble the effects arising from ordinary glucose broth cultures; (b) inflammation and necrosis of subcutaneous tissue and muscles, in which they resemble the effects produced by the bacilli themselves. Even moderate quantities of the toxic filtrate locally injected may also bring about rapid death of pigeons.

It is now possible to answer the question placed at the head of this section of our paper. The cause of death in *Bacillus welchii* infection is not a blood invasion of the microorganisms and not acid intoxication, but an intoxication with definite and very potent poisons produced in the growth of the bacilli in the tissues of the body. This poison is readily produced in broth in the test-tube in the presence of sterile non-denatured muscle. To obtain it in quantity only minimal quantities of glucose (0.1 per cent) should be added to the broth, and the incubation of the anaerobic cultures should not exceed 24 hours. The poison or toxin is a complex of an hemolysin and another poisonous body. The latter is the more toxic, since it may bring about death under conditions in which no blood destruction takes place.

### *The Toxic Product.*

*Thermolability.*—The toxicity of the filtered fluid is destroyed by heating 30 minutes at 70°C. in sealed tubes and is greatly diminished by similar heating at 62°C. The fluid subjected to the latter treatment no longer causes death in pigeons, even when large doses are injected, although a degree of necrosis of the muscles still results. A test made to determine the point indicates also that the substances exerting the toxic effects do not dialyze through collodion membranes.

*Antigenic Properties.*—The next step taken was that of determining whether the toxic product would act as an antigen. Two sets of tests were made: (a) the setting up of active immunity of the pigeon; (b) the production of an antiserum in the rabbit.

The former is difficult to accomplish because of the necrosis caused even by sublethal injections into the pectoral muscles of pigeons. However, by giving three carefully graded injections at weekly intervals, the animals may be kept in fair condition. 1 week after the last injection, the pigeons bore two lethal doses of the toxic filtrate without reaction.

The latter is accomplished with less difficulty. Large male rabbits were employed. 2 cc. of a neutralized filtrate of Culture 617 d were injected beneath the skin of the inner aspect of the thigh. This was followed by edema involving the scrotum. The edema subsided in a few days, leaving the scrotal skin necrotic and dry. 10 days after the first injection a second one was given on the opposite side. The effects were the same as the first. A third injection of 3.5 cc. was given on the right side after a similar interval. No reaction followed. The rabbit was now bled and a series of neutralizations performed, as shown in Table I. The toxic filtrate was mixed with the serum from the immunized rabbit or normal rabbit and injected immediately into the pectoral muscles of the pigeon or subcutaneously into the rabbit and guinea pig.

The table shows that the blood of a rabbit which has received three injections of a toxic filtrate from a given culture is capable of neutralizing not only that particular filtrate, but the filtrate from four other cultures as well. The neutralization is effective against the filtrate obtained from several distinct cultures and for the three species of animals—pigeon, rabbit, and guinea pig—employed.

Moreover, the neutralization is not only for the toxic substance causing inflammation and necrosis of the local tissues, but also for the specific hemolysin contained in the filtrates. This is an important point, since it controverts the notion that the blood destruction results from acids produced in course of growth of the bacilli.

*Experiment 13.*—(a) Pigeon. Red cells 4,250,000; no free nuclei. 2.10 p.m. Injected into wing vein mixture of 1 cc. of toxic filtrate of No. 617 d and 1 cc. of normal rabbit serum. 3.40 p.m. Red cells 1,312,000; many free nuclei. 3.40 p.m. Death.

(b) Pigeon. Red cells 4,500,000. 11.45 a.m. Mixture of 1 cc. of toxic filtrate of No. 617 d and 1 cc. of immune rabbit serum injected into wing vein. 1.45 p.m. Red cells 4,264,000. 10 a.m. next day. Red cells 4,300,000. No symptoms appeared.



TABLE I.

Hr. of injection.	Animal injected.	Toxic product.		Mixed with immune or normal rabbit serum.		Local reaction.	Final result.
		Quantity.	Source.				
a. m.		cc.		cc.			
9	Pigeon.	1	617 d	0.5	Immune.	None.	Survived.
9	"	1	617 d	0.5	Normal.	In 2 hrs. ex- tensive ede- ma.	Died, 2.10 p.m.
9	"	3	365 a	1.0	Immune.	None.	Survived.
9	"	3	365 a	1.0	Normal.	In 3 hrs. ex- tensive ede- ma.	Died, 4.10 p.m.
9	"	3	669 b	1.0	Immune.	None.	Survived.
9	"	3	669 b	1.0	Normal.	In 4½ hrs. mus- cle greatly swollen.	Died, 1.45 p.m.
9	"	3	P-50	1.0	Immune.	None.	Survived.
9	"	3	P-50	1.0	Normal.	In 5 hrs. mus- cle greatly swollen.	Died, 2.30 p.m.
9	"	4	386 cd	1.0	Immune.	None.	Survived.
9	"	4	386 cd	1.0	Normal.	In 5 hrs. mus- cle swollen.	Died, 2.45 p.m.
10	Rabbit.	2	617 d	1.0	Immune.	None.	Survived.
10	"	2	617 d	1.0	Normal.	Extensive scro- tal edema.	Recovered.
10	Guinea Pig A.	2	617 d	1.0	Immune.	None.	Survived.
10	" " B.	2	617 d	1.0	Normal.	Extensive swel- ling of leg and scrotum.	Necrosis. Died on 7th day.

*Neutralizing Proportions.*—The next experiment was designed to determine the minimal lethal dose of the toxic filtrate and the necessary neutralizing quantity of immune serum for that dose. This having been ascertained, an experiment was conducted to decide whether the neutralization took place equally in multiple proportions.

Pigeons were employed for the tests. The minimal lethal dose of the toxic filtrate employed proved to be 0.3 cc., from which death resulted in about 8 hours. The perfectly neutralizing quantity

of the immune serum for this dose was 0.2 cc. When mixed together and injected into the pectoral muscles no reaction followed. The minimal protective dose of the immune serum was much smaller; namely 0.05 cc. But with this dose considerable local reaction manifested itself.

The experiment with multiple proportions of toxic filtrate and immune serum was made with twenty-five doses of each. Hence 7.5 cc. of the toxic filtrate and 5.0 cc. of the immune serum were mixed and the entire volume was then injected into the breast muscles of each of two pigeons. No signs of intoxication developed, and aside from slight local edema in one of the pigeons, no symptoms whatever appeared. From this it was concluded that the toxic filtrate and antitoxic rabbit blood neutralized each other perfectly in multiples of the single doses. In this respect the two resemble the corresponding toxins and antitoxins of *Bacillus diphtheriæ* and *Bacillus tetani*.

#### *Protective and Curative Properties.*

The experiments described having clearly shown that the toxic products of the growth of *Bacillus welchii* exhibit antigenic activities and readily give rise to the formation of active antitoxic substances, the obvious next step was to determine whether the immune serum developed possessed protective and curative properties. Two sets of tests bearing on these questions have been made.

In one, vegetative bacilli have been injected into the breast muscles of pigeons mixed with or followed by the immune serum. The result is to prevent or reduce the pathogenic effects otherwise produced. Normal rabbit serum has no such power of control. These experiments will be published in detail later.

In the other, the object was to imitate conditions of natural infection in man with a view to preventing infection from arising. For this purpose, the bacilli were cultivated by Dunham's method so as to obtain spores. Bits of gauze were impregnated with the sporulating cultures and thrust into the breast muscle of the anesthetized pigeons with a small hemostat. The wound at once filled with blood and became sealed. Hence the conditions of a foreign body carrying active spores of the gas bacilli imbedded in muscle tissue

and protected from access of air as occurring in man were reproduced on a small scale. The test proved a severe one. Five pigeons were employed in a series. One only was treated with the immune serum, the other four serving as controls. This plan was adopted to remove the fallacy of an accidental survival of the treated animal. 2.0 cc. of the immune serum were injected, partly about the wound, partly in the opposite breast. The four inoculated but untreated pigeons developed typical local lesions and succumbed in 20 to 40 hours after inoculation. The treated animal never showed any local or general symptoms, survived, and the wound healed about the foreign body.

The experiments briefly reported in this section of the paper seem to possess considerable importance. They indicate, indeed, that in *Bacillus welchii* infection in nature the development of the spores into vegetative bacilli may be prevented by a protective inoculation of an antitoxic serum, and also that the vegetative bacilli may be deprived by such a serum of their toxic products, which now appear to be their real offensive instrument. We are confronted, therefore, not only with a new point of view regarding the manner of the pathogenic action of the Welch group of bacilli but also with a new means of combating their pathogenic effects.

#### DISCUSSION.

The experiments presented appear to admit of one interpretation only; namely, that the Welch bacilli, under suitable conditions of growth, produce an active exotoxin, to which their pathogenic effects are ascribable. The toxic product, moreover, acts upon the local tissues and the blood in a manner identical with the action of the cultures. With the toxic product animals may be immunized actively and yield an immune serum which neutralizes the toxin perfectly and in multiple proportion. The toxic bodies would seem to be at least two in number: one causing blood destruction, hence an hemolysin, and the other acting locally on the tissues and blood vessels, causing edema and necrosis and probably exerting general toxic action in addition. The part each plays in bringing about the lethal effect seems to be determined by the manner of inoculation: to bring out the

hemolytic action intravenous injection is indicated; to bring out the locally destructive action, subcutaneous or intramuscular injection is required.

This conception of the manner of pathogenic action of the Welch bacilli is totally different from any view previously held. It is true that others have attributed the general symptoms in *Bacillus welchii* infection to an intoxication; but the poisoning meant was one ascribed on the one hand to decomposition products of the infected tissues (E. Fraenkel) and on the other to ordinary endotoxin absorption (Metchnikoff, Korentchewsky, Kamen, Herter, Passini). Other views have also been expressed and insisted upon, and they would ascribe the locally destructive effects of the bacilli to mechanical action (Taylor) or to the production of fatty acids which also through the setting up of an acidosis bring about a lethal termination (McCampbell, Stewart and West, Wright).

Reference will be made only to the views expressed by recent writers who have encountered gaseous gangrene in connection with gun-shot wounds of the great war. Thus Weinberg,<sup>6</sup> who believes that the gas-producing bacilli do not cause the gangrene, but that the condition precedes the infection, has obtained toxic and antitoxic products from various anaerobic bacteria isolated from gangrenous wounds. With *Bacillus perfringens* (of the *Bacillus welchii* group) he has prepared an antibacterial serum, but he failed to detect either the exotoxin or its corresponding antiserum.

Kenneth Taylor<sup>7</sup> considers that gaseous gangrene is the result of the mechanical action of the gas produced in a local focus of developing saprophytic bacteria. He specifically draws the distinction between *Bacillus tetani* and *Bacillus welchii* infections, since with the former the toxin is the active factor, while with the latter the mechanical effect of the gas is paramount. The mechanical process he conceives to be as follows: *Bacillus welchii* attacks the carbohydrates of muscular tissue and produces a large volume of gas, which, being unable to escape from the tissues, exerts pressure upon the blood vessels, impeding the circulation so that necrosis results. The necrotic tissue is invaded by putrefactive bacilli which disorganize it.

Sir Almroth Wright<sup>8</sup> holds that *Bacillus welchii* operates through the production of an acid condition of the blood and tissues, through which the antitrypsin is diminished. Because of this diminution, tryptic digestion of the proteins is permitted and the bacilli are thus provided with a highly favorable medium of growth, so that multiplication becomes explosive in nature. The intoxication following is in fact an acidemia.

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<sup>6</sup> Weinberg, M., *Proc. Roy. Soc. Med.*, 1916, ix, Occas. Lect., 119.

<sup>7</sup> Taylor, K., *Bull. Johns Hopkins Hosp.*, 1916, xxvi, 297; *J. Path. and Bacteriol.*, 1916, xx, 384.

<sup>8</sup> Wright, A. E., *Proc. Roy. Soc. Med.*, 1916-17, x, Occas. Lect., 1.

Conradi and Bieling<sup>9</sup> distinguish two phases of action of the bacilli. In the first or fermentation phase, the carbohydrates are attacked, and lactic, butyric, propionic, and succinic acids are formed, which are the immediate causes of the edema and necrosis of the tissues. In the second or saprophytic stage, the spore-bearing organisms appear and appropriate the dead tissue, giving rise to putrefaction and consequent intoxication.

These brief extracts readily indicate not only the wide diversity of opinion held by recent students of the pathogenesis of gas bacillus infection in man, but show also how remote the conceptions are from that of a specific pathogenetic process, due to the action of particular toxic substances, which is the basis of the conviction derived from the experiments described by us. According to our view, infection by *Bacillus welchii*, like infection by *Bacillus tetani*, essentially resolves itself into an intoxication, in which an exotoxin yielded by the multiplying organisms constitutes the chief danger. The two conditions differ, however, with respect to the local effects produced on the tissues, since the tetanus toxin does not possess inflammatory and necrotizing properties. The Welch bacilli, therefore, grow more abundantly and produce wide destruction of tissue, in which process they are soon assisted by the usual pyogenic microorganisms, which quickly obtain a foothold in the disorganized structures.

#### SUMMARY.

Five cultures of *Bacillus welchii* have been studied and compared. Four came from infected wounds in the western theatre of war, and one was obtained from a personal article of clothing. Each culture possesses the essential characteristics ascribed to that group of bacteria.

The infectious processes caused by the five cultures in rabbits, guinea pigs, and pigeons, are local in character; and very few or no bacilli enter or are found in the general blood stream during life or immediately after death.

Glucose broth cultures, injected intravenously, are fatal to rabbits. Death occurs almost immediately or after a few hours. Agglutinative bacterial emboli have been ruled out as the cause of death,

<sup>9</sup> Conradi, H., and Bieling, R., *Münch. med. Woch.*, 1916, lxiii, 1608.

as has been an acid intoxication. The fluid part of the culture acts in the same manner as the full culture and irrespective of neutralization with sodium hydroxide.

The full cultures and supernatant fluid are hemolytic when injected directly into the circulation of rabbits and pigeons, and the acute death produced may be ascribed to a massive destruction of red corpuscles. The passage of the fluid portion of glucose broth cultures through Berkefeld filters reduces materially the hemolytic and poisonous effects.

Cultures of the Welch bacilli in plain broth to which sterile pigeon or rabbit muscle is added are highly toxic, and the toxicity is not noticeably diminished by Berkefeld filtration. The filtrates are hemolytic when injected intravenously and inflaming and necrotizing when injected subcutaneously and intramuscularly. The local lesions produced in the breast muscles of the pigeon closely resemble those caused by infection with the bacilli.

The toxicity of these filtrates is not affected by neutralization with sodium hydroxide, but is materially reduced by heating to 62°C. and entirely removed by heating to 70°C. for 30 minutes.

Successive injections of carefully graded doses of this toxic filtrate in pigeons and rabbits give rise to active immunity. The blood taken from the immunized rabbits is capable of neutralizing the toxic filtrate *in vivo* and *in vitro*. The filtrate has therefore been designated as toxin and the immune serum as antitoxin.

The antitoxin neutralizes the toxin in multiple proportions. Hence the latter would seem to possess the properties of an exotoxin. Moreover, it neutralizes the hemolytic as well as the locally injurious toxic constituent.

Antitoxic serum prepared from a given culture of *Bacillus welchii* is neutralizing for the toxins yielded by the other four cultures of that microorganism.

The antitoxin is protective and curative against infection with the spore and the vegetative stages of *Bacillus welchii* in pigeons. The limits of the protective and curative action are now under investigation.

## STUDIES IN GLOMERULONEPHRITIS.

### I. A QUANTITATIVE STUDY OF THE REACTION OF THE KIDNEY TO DIPHTHERIA TOXIN.

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PLATES 9 AND 10.

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In investigating the pathogenesis of acute diffuse glomerulonephritis, it was thought that it might be of value to test the hypothesis that a soluble poison circulating in an active state in the blood will exert a more intense action in the glomerulus than in other tissues, because at this point water is lost from the blood, and any poison present, provided it was not excreted with the water, would necessarily become concentrated and its powers of injury intensified. Such a condition of increased concentration may indeed be assumed *a priori* to occur, the question being whether it is great enough to produce recognizably heightened damage.

For the purpose of testing this hypothesis, diphtheria toxin was selected as answering the experimental requirements. Injected into the blood of non-immune animals it is actively and directly toxic and it does not pass through the glomerular filter into the urine.

Our problem, then, is to compare the effects of different dosages of this poison, and to determine whether small amounts of toxin, injected intravenously, will produce glomerular lesions before demonstrable injury occurs elsewhere, and whether larger amounts produce damage proportionally greater in the tufts than in other parts of the renal tissue. With full dosages, of course, the well recognized picture of a pannephritis (or pannephrosis) is to be expected.

The studies of Oertel and of Councilman, Mallory, and Pearce are perhaps the most important ones treating of the disease in man, and the papers of Wade, who first described nephritis in diphtheria, Wagner, Brault, Fürbringer, Fischl,

Schrakamp, von Kahliden, Bernhard and Felsenthal, Reiche, and Katzenstein may also be mentioned. These studies make it clear that both tubular degeneration and glomerular alterations occur in the course of human diphtheria. The glomerular lesions consist of swelling of the endothelium, dilatation of the loops, various degrees of karyorrhexis of the endothelial nuclei and, in more advanced cases, hyalinization and obliteration of the tuft occur.

Experimental investigations have been made by Roux and Yersin, Spronck, Babes, von Kahliden, Enriquez and Hallion, Baldassari, Welch and Flexner, Flexner, Schlayer and Hedinger, Takayasu, Lyon, Karsner and Denis, Pearce and Eisenbrey, Frothingham, and Bailey. These investigators have presented anatomical or functional evidence of glomerular as well as of tubular injury in experimental diphtheria or diphtherial intoxication. It is clear from a consideration of the work of Baldassari and especially of Flexner that diphtheria toxin is a general cellular poison without specific chemical affinity for any one type of tissue.

Charrin and Moussu showed that diphtheria toxin does not diffuse through animal membranes; Rodet and Guéchoff, that it does not diffuse through collodion;<sup>1</sup> Dzerzhgovski and Onufrovich, that it does not diffuse through the renal tissues when the excised kidney is perfused with a 10 per cent solution in blood; and Bomstein, that it is not excreted into the urine.

Diphtheria toxin, therefore, appears to be well suited to the purposes of the present experiments.

#### EXPERIMENTAL.

The toxin<sup>2</sup> employed had a strength of 200 minimum lethal doses per cc. when tested April 18, 1916. No evidence of deterioration was noted during the present series of experiments. All injections were made intravenously in quantities of 1 cc., made up in 0.85 per cent salt solution. The rabbits were in most cases young, weighing between 1,000 and 1,500 gm., and were, in so far as external signs showed, in good health. The animals that did not die spontaneously were killed by a blow on the neck and immediately autopsied. The tissues were fixed in Orth's solution with a 5 per cent addition of glacial acetic acid, and embedded in paraffin. Sections were stained by Van Gieson's method, with hematoxylin and eosin, and in a few

<sup>1</sup> Roux and Yersin found some diffusion through parchment paper. The pinholes which can be readily seen in most samples of parchment paper with a hand lens probably explain this discrepancy.

<sup>2</sup> The toxin was prepared at the Cutter Laboratory of Berkeley, California, for use in the Schick test.



instances by Weigert's method for fibrin. Frozen sections were not made. The results are shown in the following table and protocols.

TABLE I.

Rabbit No.	Date of injection.	Weight of rabbit.	Toxin injected.	Dosage per kilo of body weight.	Length of life after injection.	Killed or died.
	1916	gm.	cc.	cc.	days	
1	Sept. 6	1,475	0.001	0.00068	3	K.
2	" 6	1,475	0.001	0.00068	3	"
3	" 6	1,475	0.001	0.00068	3	"
4	" 6	1,420	0.001	0.0007	3	"
5	May 2	1,000	0.001	0.001	3	D.
6	Sept. 6	1,500	0.002	0.0013	3	K.
7	" 6	1,450	0.002	0.0014	3	"
8	" 6	1,400	0.002	0.0014	3	"
9	" 6	1,340	0.002	0.0015	3	"
10	" 6	1,340	0.003	0.0022	3	"
11	" 6	1,360	0.003	0.0022	3	"
12	May 2	1,300	0.003	0.0023	10	"
13	Sept. 6	1,280	0.003	0.0023	3	"
14	" 6	1,225	0.003	0.0024	3	"
15	Apr. 27	1,250	0.005	0.004	1½	D.
16	Sept. 1	1,530	0.010	0.0065	2	"
17	" 1	1,530	0.010	0.0065	2	"
18	" 1	1,500	0.010	0.0067	2	"
19	" 1	1,500	0.010	0.0067	2	"
20	" 1	1,450	0.010	0.0069	½	"
21	" 1	1,330	0.010	0.0075	2	"
22	" 1	1,420	0.020	0.0141	2	"
23	" 1	1,300	0.020	0.0154	1	"

## PROTOCOLS.

*Rabbit 1.*—Dosage 0.00068 cc. of toxin per kilo. Combined weight of kidneys 11.9 gm. The macroscopic and microscopic appearance is normal.

*Rabbit 2.*—Dosage 0.00068 cc. per kilo. Combined weight of kidneys 13.7 gm. Macroscopically the cortex and outer part of the medulla are slightly congested. Microscopically normal except for a slight amount of granular exudate in the capsular space.

*Rabbit 3.*—Dosage 0.00068 cc. per kilo. Combined weight of kidneys 12.3 gm. Macroscopically normal. Microscopically the glomeruli are somewhat larger than normal; the endothelial cells are slightly increased in number, and the nuclei of the endothelium both in the tufts and the intertubular capillaries are occasionally pyknotic.

*Rabbit 4.*—Dosage 0.0007 cc. per kilo. Combined weight of kidneys 16 gm. Macroscopically the boundary zone of the medulla is congested. Microscopically the endothelial cells of the tufts are somewhat increased in number.

*Rabbit 5.*—Dosage 0.001 cc. per kilo. Combined weight of kidneys 9.5 gm. Macroscopically some congestion of boundary zone. Microscopically some increase of the endothelial cells of the tufts, the nuclei of which are occasionally pyknotic. Occasional pyknotic nuclei also in the tubular epithelium. A distinct increase of leukocytes in the tufts.

*Rabbit 6.*—Dosage 0.0013 cc. per kilo. Macroscopically the boundary zone of the medulla is congested. Microscopically the glomeruli are in part enlarged and most of them are greatly distended with blood. The endothelial nuclei are frequently pyknotic, and there is a considerable increase in the number of endothelial cells in the tufts. The leukocytes in the tufts are somewhat increased in number.

*Rabbit 7.*—Dosage 0.0014 cc. per kilo. Combined weight of kidneys 13.5 gm. Macroscopically moderate congestion of the medulla. Microscopically the glomeruli show swelling of the endothelial cytoplasm, some loops are distended and empty, many of the nuclei are pyknotic, and there is an increase in the number of endothelial cells (Figs. 1 and 2).

*Rabbit 8.*—Dosage 0.0014 cc. per kilo. Combined weight of kidneys 16 gm. Macroscopically normal. Microscopically some of the glomeruli are much enlarged and the endothelium generally is much swollen, with many empty loops; the nuclei are moderately increased in number and many are pyknotic or misshapen. There are some desquamated epithelial cells in the capsular space and in the tubules there are a good many hyaline casts.

*Rabbit 9.*—Dosage 0.0015 cc. per kilo. Combined weight of kidneys 10.8 gm. Macroscopically normal. Microscopically marked increase in the number of endothelial nuclei of tufts, a considerable number of which are pyknotic. Moderate endothelial swelling. Occasional glomeruli are much enlarged.

*Rabbit 10.*—Dosage 0.0022 cc. per kilo. Combined weight of kidneys 15.5 gm. Macroscopically moderate general congestion. Microscopically marked swelling of the glomerular endothelium with dilatation of the loops; many pyknotic nuclei, and slight increase in the number of cells. A considerable number of hyaline casts. The epithelium of the tubules is granular in appearance, but there is no desquamation and no pyknosis of the nuclei.

*Rabbit 11.*—Dosage 0.0022 cc. per kilo. Combined weight of kidneys 15 gm. Macroscopically nothing abnormal. Microscopically marked increase in the number of the endothelial cells of the tufts, of which the cytoplasm is swollen and the nuclei are pyknotic. Some dilatation of the loops. In places, some stratification of the parietal epithelium of the capsule of Bowman is seen. The tubular epithelium contains pyknotic nuclei in places; the cytoplasm is granular and shows beginning desquamation. Some hyaline casts.

*Rabbit 12.*—Dosage 0.0023 cc. per kilo. Combined weight of kidneys 12 gm. Macroscopically moderate general congestion. Microscopically swelling and

numerical increase of the endothelial cells of tufts, with occasional pyknotic nuclei. Changes are not so marked as in preceding.

*Rabbit 13.*—Dosage 0.0023 cc. per kilo. Combined weight of kidneys 9.6 gm. Macroscopically general pallor. Microscopically glomeruli large with dilated loops and swollen endothelium, the cells being somewhat increased in number with occasional pyknotic nuclei. The tubular epithelium shows moderate cloudy swelling.

*Rabbit 14.*—Dosage 0.0024 cc. per kilo. Combined weight of kidneys 13.8 gm. Macroscopically moderate general congestion. Microscopically the glomeruli are very large with many dilated empty loops (Fig. 3). The endothelium is greatly swollen. The capsular space is almost obliterated. There are a few small intraglomerular hemorrhages. Considerable desquamation of the tubular epithelium, many of the nuclei of which are pyknotic, misshapen, and in places extruded into the lumen. There are many hyaline casts.

*Rabbit 15.*—Dosage 0.004 cc. per kilo. Combined weight of kidneys 22 gm. Macroscopically they are much swollen and intensely congested; the cortical surface shows whitish, poorly defined patches, which on the cut surface are seen as radial bands extending into the medulla; probably the medullary rays contrasting with the deeply congested cortical labyrinths. Microscopically the majority of glomeruli are hemorrhagic and converted into cysts of blood which is mainly fresh but in places shows beginning coagulation (Fig. 4). These hemorrhages are mostly confined within the intact epithelium of the visceral layer of Bowman's capsule, but in places the latter has ruptured and flooded the capsular space and the proximal tubules with red blood cells. The glomerular nuclei that are still recognizable are pyknotic or fragmented. In the tubules the epithelium is in places granular and the nuclei are occasionally pyknotic but the evidences of severe damage are much less than in the glomeruli. There is no evidence of repair.

*Rabbit 16.*—Dosage 0.0065 cc. per kilo. Combined weight of kidneys 26 gm. Macroscopically the kidneys are greatly enlarged and intensely congested with several small superficial hemorrhages under the capsule. Microscopically the appearance is practically that of Rabbit 15, except that more of the hemorrhages are coagulated, showing masses of fibrin, and the epithelial degeneration in the tubules is more advanced (Figs. 5 and 6).

*Rabbits 17 and 18.*—Practically the same picture as No. 15.

*Rabbit 19.*—Dosage 0.0067 cc. per kilo. Combined weight of kidneys 20 gm. Macroscopically the kidneys are dark purple in color, with several subcapsular hemorrhages; the cut surface shows a much thickened cortex in which there are many small hemorrhages, and the glomeruli are seen as fine red points. The medullary rays are paler than the labyrinths. Microscopically the hemorrhages are seen to be from the intertubular capillaries both in the cortex and the outer medullary zone. In the tufts, the glomerular endothelium has undergone acute degeneration, the lumina being filled with an amorphous granular material, and the nuclei are all pyknotic or fragmented except the large epithelial nuclei, which

in most places appear to be little damaged. There is marked coagulation necrosis of the tubular epithelium, especially of the proximal tubules, many of which show extruded pyknotic nuclei and desquamated epithelium in the lumen (Fig. 7).

*Rabbit 20.*—(Died 12 hours after injection.) Dosage 0.0069 cc. per kilo. Combined weight of kidneys 19 gm. Macroscopically they are swollen and dark purple in color without gross hemorrhages. Microscopically there is extreme swelling of the glomerular endothelium and the loops are dilated and empty. The nuclei are pyknotic. The epithelium of the tubules shows coagulation necrosis with the nuclei pyknotic. The intertubular capillaries are greatly dilated.

*Rabbit 21.*—Dosage 0.0075 cc. per kilo. Combined weight of kidneys 19 gm. Macroscopically the kidneys are deep purple with congestion, but there are no gross hemorrhages. Microscopically the glomerular endothelium is in places swollen, but as a rule it is definitely necrotic and disintegrated, the lumina being filled with finely granular debris. The nuclei are pyknotic and fragmented. There are occasional intraglomerular hemorrhages. The epithelial degeneration of the tubules is marked, but no formed casts are present.

*Rabbit 22.*—Dosage 0.0141 cc. per kilo. Combined weight of kidneys 16 gm. Macroscopically intense congestion. Microscopically there is disintegration of the capillary endothelium in the tufts and in the intertubular capillaries with thrombi of finely granular material. There are many greatly dilated empty loops and a large amount of serous exudate (Fig. 8) containing in places red blood cells. Marked epithelial degeneration in the tubules.

*Rabbit 23.*—Dosage 0.0154 cc. per kilo. Combined weight of kidneys 15 gm. Macroscopically intense congestion. Microscopically extreme swelling of the glomerular endothelium with beginning necrosis, and thrombosis of the loops. Many of the loops are dilated and empty. The epithelial degeneration is not advanced, but the cytoplasm appears somewhat granular and occasional pyknotic nuclei are seen. The rabbit died 24 hours after injection.

The changes described in the protocols indicate, in spite of slight differences at the same level of dosage, that the severity of the lesion is roughly proportional to the size of the dose administered. It is evident that the primary change is in the endothelium of the tuft. It is possible that a simultaneous alteration of the intertubular capillaries occurs, but is difficult to detect, because of the intimate relation between them and the walls of the renal lymphatics. Even hemorrhage from these capillaries is hard to recognize, since the escaping blood follows the lines of cleavage which closely correspond to the normal path of the small vessels. However, it was found that the small flat nuclei between the tubules were frequently pyknotic and this occurred even in the rabbits receiving the smallest doses. With

larger doses, the capillaries, especially in the inner cortical and outer medullary zones, were found to be greatly dilated and full of blood.

With dosages approximating 0.0007 cc. of diphtheria toxin per kilo in rabbits very slight changes of any kind are produced in the kidney, though occasional deeply staining endothelial nuclei and a slight increase of these nuclei in the glomeruli give evidence that some damage has been inflicted. With dosages between 0.001 and 0.0015 cc. per kilo, changes of increasing severity are found, consisting of evidences of both nuclear and protoplasmic irritation and injury such as multiplication of the nuclei and swelling of the cytoplasm of the glomerular endothelium. With these doses evidence of epithelial injury in the tubules is present, but not striking. With dosages over 0.002 cc. per kilo the glomerular changes become more severe as shown by marked endothelial swelling, extensive pyknosis of the nuclei, and marked enlargement of the tufts with dilatation of those which remain pervious. At this stage, degeneration of the tubular epithelium is indicated by pyknosis and karyorrhexis, desquamation of the cells, and the formation of numerous casts, but even in the parenchyma the presence of pyknotic nuclei in the intertubular capillaries suggests the possibility of primary vascular damage. In the glomeruli it is sometimes possible to distinguish the epithelial nuclei of the visceral and parietal layers of the capsule apparently unharmed, while in the same tuft the endothelial nuclei show advanced injury. With doses of 0.004 cc. per kilo and more, the glomerular capillaries rupture and globular hemorrhages confined by the epithelial syncytium of the capsule are seen. In some instances the capsule, too, gives way and the capsular space and proximal tubules are found to be flooded with newly extravasated blood cells.

In so far as diphtheria toxin causes glomerular hemorrhages, it is apparently by simple rupture of the capillaries, not by diapedesis. The lesion sometimes observed in human hemorrhagic glomerular nephritis, in which fibrin, red blood cells, and leukocytes exude into the capsular space, is notably absent. At most, the only real exudate into the space is a homogeneous, albuminous material, observed in one kidney of the present series, though desquamated epithelial cells from the capsule may give a superficial resemblance to a true exudate.

With doses of 0.0065 cc. per kilo and more, such hemorrhages are less constant, and instead we find thrombotic masses within the necrotic capillaries. The nature of these masses is somewhat difficult to determine. They do not take the Weigert stain for fibrin, but with Van Gieson's stain they are brownish in color. Under a low power objective they form interlacing and branching figures about the size and conformation of the glomerular capillaries. Closely examined with the high power objective they are seen to consist of very finely granular amorphous material usually completely occluding the capillaries but in some places lying in the center of the lumen. It seems probable that the material is the necrotic and desquamated cytoplasm of the endothelium, a supposition which is strengthened by the presence in the material of faintly staining or pyknotic and often fragmented nuclei. It is possible also that some of this material may be embolic, derived from the degenerated intimal endothelium of the afferents, a process observed by von Kahliden in some cases of human diphtherial nephritis. With these high dosages there is evidence of rapid death of the whole kidney, with pyknotic and fragmented nuclei, and granular, desquamated, or coagulated cytoplasm. Such doses are usually fatal within 48 hours. The maximum effect is obtained apparently with doses of about 0.008 cc. per kilo, the pathological changes resulting from 0.015 cc. per kilo being nearly the same as with the former amount.

Leukocytes within the vessels and occasionally surrounding the vessels or the glomerular capsule were sometimes found but, as the protocols show, this phenomenon bears no relation to the dosage of toxin and should probably be regarded as due to other causes. A possible rôle of the leukocytes in these kidneys is to remove necrotic fragments by phagocytosis. A mobilization of leukocytes as a result of leukotactic action by the toxin is not shown by the present series of experiments.

#### DISCUSSION.

It is not necessary to assume a specific affinity of diphtheria toxin for endothelium in order to account for the vascular injury found in the above experiments. Babes and later Baldassari determined that the primary action of diphtheria toxin is upon the nuclei of the body

cells in general. As regards the kidney it is possible that the small amount of cytoplasm of the flat endothelial cells acts as a less efficient buffer for the nucleus against the attack of the toxin than the abundant cytoplasm of the tubular epithelium, but a simpler explanation is that the toxin, being brought to the kidney by the blood stream attacks the tissue with which it comes in direct contact; namely, the endothelium of the vessels.

As regards the location of severe injury in the tuft the experimental findings stand in fairly close agreement with the theoretical expectation that the glomerulus should show an especially severe grade of injury because the circulating toxin reaches at this point a relatively high concentration. It was actually found that the earliest recognizable alteration, and with larger doses, the severest lesions were in the glomeruli, and perhaps the intertubular capillaries, and that these lesions were essentially endothelial.

These endothelial glomerular lesions were observed in the present series in all degrees of intensity, progressing through the following stages: (1) cellular proliferation; (2) swelling of the cytoplasm with partial or total occlusion of the capillaries and beginning degeneration of the nuclei; (3) rupture of the capillaries with hemorrhage; (4) necrosis and disintegration of the cytoplasm with blockage of the capillaries with protoplasmic debris.

It has been shown by Charrin and Moussu that diphtheria toxin does not diffuse through animal membranes, and by Rodet and Guéchoff that it does not diffuse through collodion, and we may infer that during life physical dialysis of toxin through the glomerulus and tubules does not occur. To explain the action of diphtheria toxin on the tubular epithelium we have to assume either a selective activity of the tubular cells or an effect secondary to vascular injury. That the first of these assumptions is unlikely is shown by the fact established by Bomstein that toxin is not excreted into the urine, and by the findings of Dzerzhgovski and Onufrovich that toxin is not retained by the kidney in perfusion experiments. Anatomically the secondary nature of the epithelial lesions is indicated by the fact that with small doses of toxin glomerular lesions are recognizable before tubular degeneration appears and that with doses of medium size the glomerular lesions are much severer than the tubular. In short, endothelial injury is predominant.

A point deserving emphasis is the slight anatomical evidence of excretion of toxin from the tufts. This is shown by the small amount of damage to Bowman's capsule and the small quantity of exudate in the capsular space. It was notable in many kidneys that, while the endothelial cells were necrotic and disorganized, the epithelial cells were surprisingly well preserved and the parietal layer of the capsule little altered. A striking confirmation of the truth of this observation was the frequent occurrence of hemorrhages obliterating practically the entire capillary structure of the tuft, but confined within the unruptured epithelial syncytium of the visceral layer of Bowman's capsule. Only with relatively enormous doses of toxin and then in one case only was there a marked exudate in the capsular space such as we find in the so called extracapillary form of glomerulonephritis. To explain the pathogenesis of the latter form of renal disease, we must perhaps adduce other factors which possibly call into action some specific excretory activity of the glomerular epithelium.

The lesions described in the present experiments, at least those obtained with subhemorrhagic doses of toxin, with their evidence of injury within the tuft are strongly reminiscent of those described by Volhard and Fahr under the name of intracapillary glomerulonephritis. They say:

"The glomeruli are strikingly large. . . . containing little blood, and the cellular elements within the tufts are enormously increased. The loops are swollen and often become adherent and hyalinized. . . . In the capsular space occasional desquamated epithelium, a little coagulated albumin and here and there red blood cells are seen. . . . but usually the space is empty."

We shall not in my opinion, be far from the truth if we apply our observations and deductions to the pathogenesis of the intracapillary form of glomerulonephritis in general, and assume that this form of disease is the result of the concentration, due to loss of water in the tuft, of poisons circulating in an active state in the blood. It seems highly probable that such a process plays at the least a major part in the production of these lesions.



## SUMMARY.

1. Diphtheria toxin of known strength, injected intravenously into rabbits in single doses, produces lesions which are first evident in the endothelium of the tufts, and possibly in the intertubular capillaries.

2. The lesions of greatest severity with medium doses are also found in the tufts.

3. Evidence is presented to show that epithelial damage in the tufts and especially in the tubules is secondary to the vascular injury.

4. The severity of the lesions was roughly proportional to the amount of toxin injected. Hemorrhages did not occur with doses of less than 0.0023 cc. of toxin per kilo of body weight and were not extensive with doses of less than 0.004 cc. per kilo.

5. It is suggested that the localization of lesions in the tufts is due to the concentration of toxin in the blood that follows the loss of water at this point.

6. It is further suggested that this inference may be extended to explain the pathogenesis of the so called intracapillary form of glomerulonephritis.

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## EXPLANATION OF PLATES.

## PLATE 9.

FIG. 1. Rabbit 7. Leitz obj. 6. Dosage 0.0014 cc. of toxin per kilo. Glomerulus showing marked increase of endothelial nuclei. The tubules are practically normal.

FIG. 2. Rabbit 7. Oil immersion. Swollen endothelial cells in glomerulus. The capillaries are still patent.

FIG. 3. Rabbit 14. Leitz obj. 6. Dosage 0.0024 cc. per kilo. Dilated empty glomerular capillaries.

FIG. 4. Rabbit 15. Leitz obj. 6. Dosage 0.004 cc. per kilo. Large intra-glomerular hemorrhage. Erythrocytes and desquamated epithelium in capsular space, and erythrocytes in proximal convoluted tubule (to left).

## PLATE 10.

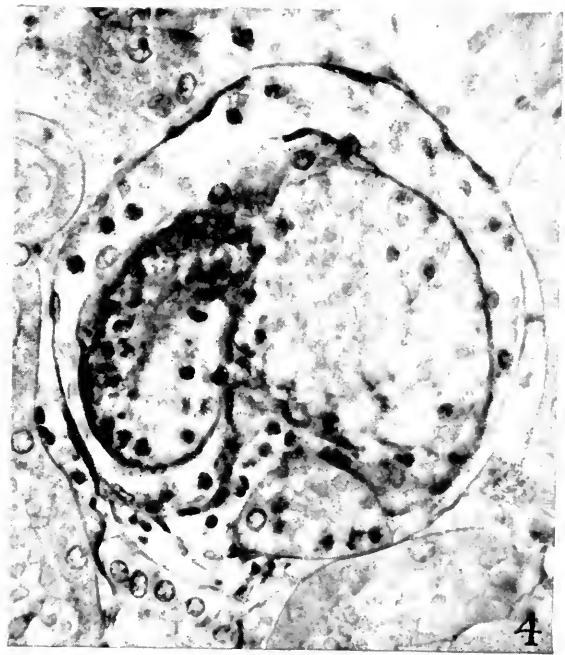
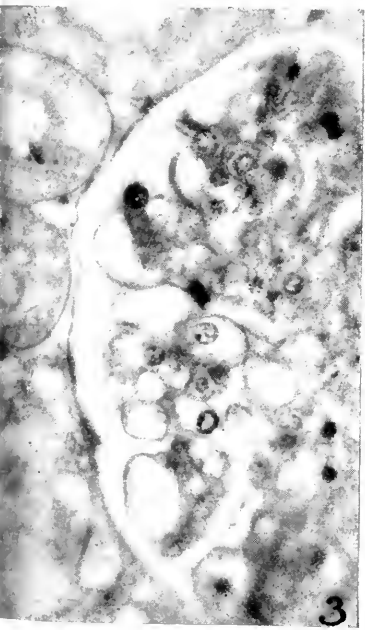
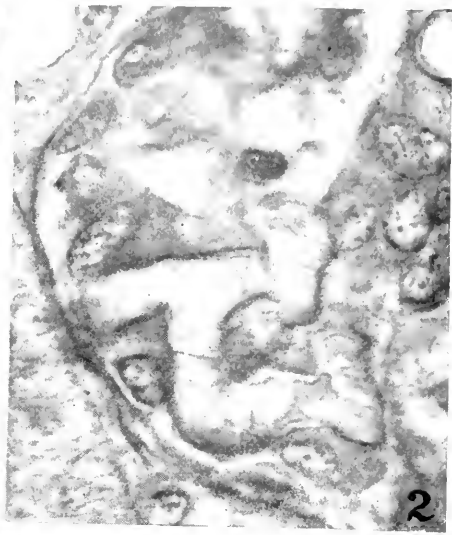
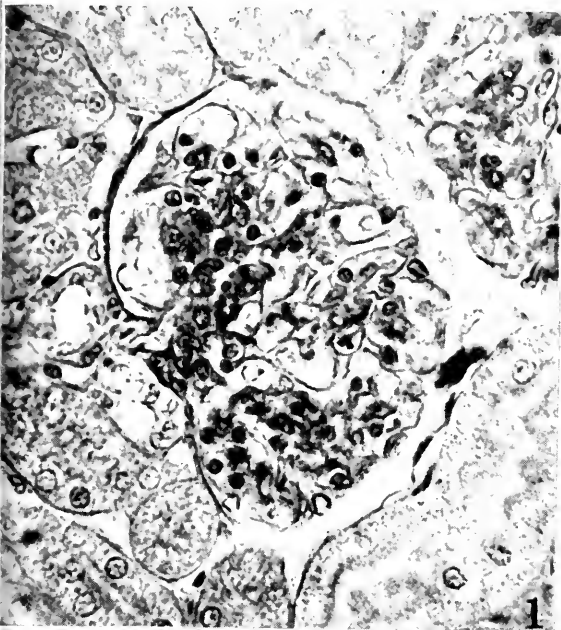
FIG. 5. Rabbit 16. Leitz obj. 6. Dosage 0.0065 cc. per kilo. Karyorrhexis and pyknosis of nuclei of tubules. The epithelium is beginning to desquamate into the lumen. Nuclei found between the tubules are also pyknotic.

FIG. 6. Rabbit 16. Leitz obj. 3. Afferent to a glomerulus containing masses of fibrin. The nuclei throughout the field are pyknotic.

FIG. 7. Rabbit 19. Leitz obj. 6. Dosage 0.0067 cc. per kilo. Tubules showing desquamation and beginning cast formation. Several nuclei show pyknosis or fragmentation.

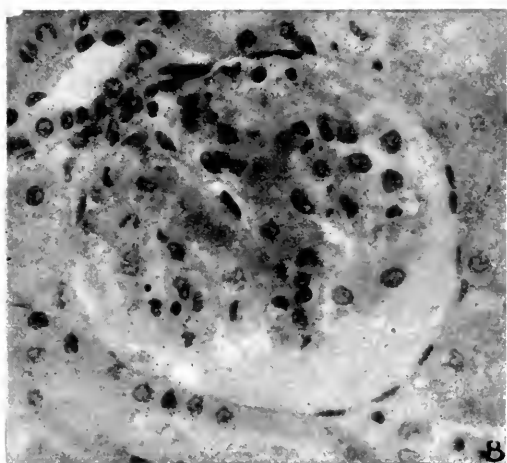
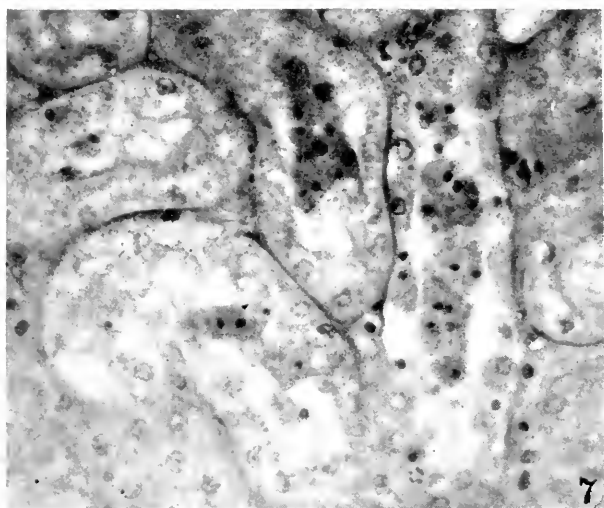
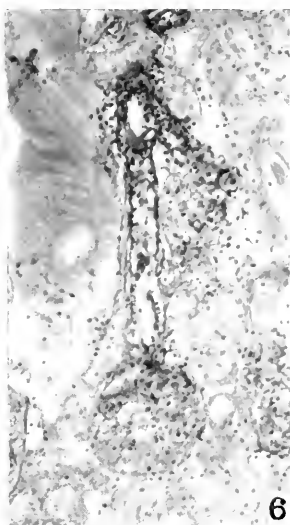
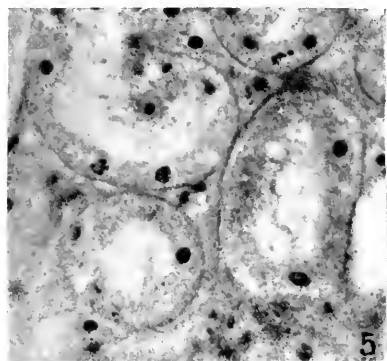
FIG. 8. Rabbit 22. Leitz obj. 6. Dosage 0.0141 cc. per kilo. The endothelium is swollen and necrotic, obliterating the capillary lumina. The nuclei are pyknotic and misshapen. The capsular space is filled with a homogeneous serous exudate. The epithelium of the capsule (parietal layer) is *in situ* and apparently little affected.





(Faber: Studies in glomerulonephritis. 1.)









## STUDIES IN GLOMERULONEPHRITIS.

### II. A FORM OF ACUTE GLOMERULONEPHRITIS PRODUCED WITH DIPHThERIA TOXIN AND BACILLUS COLI.

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PLATES 11 AND 12.

(Received for publication, March 16, 1917.)

In a previous communication it was stated that a type of glomerular injury corresponding in a general way to the intracapillary form of glomerulonephritis of Volhard and Fahr was produced by intravenous injections of diphtheria toxin and that the grade of injury was proportional to the amount of toxin introduced. Since, as a result of this knowledge, it is possible to estimate the amount of injury which can be expected to follow the injection of a given amount of toxin, it was believed that the effect of living pathogenic bacteria on a kidney already damaged to a known extent by toxin could also be correctly estimated, and that such a study might throw light on the relationship of infection to glomerulonephritis. It will be shown in a future communication that it was found impossible to produce marked glomerular lesions with bacteria alone.

#### EXPERIMENTAL.

In the following experiments *Bacillus coli communior* was selected because it is highly pathogenic for rabbits and belongs to that group of organisms which evince a special susceptibility to lysis. From our knowledge of this group we appear to be justified in ascribing a part, at least, of the pathogenic action to the poisonous groups split from their proteins during bacteriolysis.

TABLE I.  
Series A. *Injection of Diphtheria Toxin and of Colon Bacillus.*

Rabbit No.	Dose of toxin Weight	Dose per kilo-gram	Interval days	Dose of colon bacilli. cc.	Died or killed.	Length of life after second injection.	Combined weight of kidneys. gm.	Renal lesions.
1*	$\frac{0.004}{1300}$	0.0031	3	0.1	D.	36 hrs.	17.0	Many recent intraglomerular hemorrhages. Great leukocytic infiltration of tufts. Occasional masses of red blood cells in tubules. Tubular epithelium nearly normal.
2*	$\frac{0.004}{1200}$	0.0033	3	0.1	"	7 days.	16.4	Large rings and crescents of fibrin in capsular spaces and between loops in the tufts. Red blood cells in masses in a few capsular spaces. Moderate epithelial degeneration of tubules.
3*	$\frac{0.005}{1250}$	0.004	1	0.1	K.	6	19.0	Many intraglomerular hemorrhages and fibrin masses. Extreme capsular exudate, fibrin, albumin, and red blood cells. Hyaline and blood casts. Only slight tubular changes.
4*	$\frac{0.005}{1250}$	0.004	3	0.1	D.	48 hrs.	13.5	Many recent hemorrhages and numerous leukocytes in tufts. Some serous and cellular exudate in capsular spaces. Slight tubular degeneration.
5*	$\frac{0.005}{1100}$	0.0045	3	0.1	"	12 "	9.8	Glomeruli, swelling of endothelium, many leukocytes, especially in peripheral loops, much granular exudate, and occasional red blood cells in capsular space.

6	$\frac{0.001}{1200}$	0.0008	1	0.1	K.	4 days.	12.9	Wedge-shaped infarcts surrounded by zone of hemorrhage and leukocytic infiltration. Glomeruli, some large with empty loops; some contain intraglomerular hemorrhages. Some show fibrin, desquamated epithelium, red blood cells, and white blood cells in capsular space. Many hyaline casts, some giving hemoglobin stain.
7	$\frac{0.001}{750}$	0.0013	1	0.01	D.	20 min.	7.0	No striking abnormalities.
8	$\frac{0.001}{750}$	0.0013	1	0.01	"	4 days.	12.0	Extreme reaction. Masses of fibrin or fibrin and red blood cells in the tufts and capsular space. Some fresh hemorrhages. Many hyaline casts. Many leukocytes in the tufts. Occasional serous exudate in capsular space.
9	$\frac{0.002}{1300}$	0.0015	1	0.1	"	3 "	18.9	Intraglomerular hemorrhages. Red and white blood cells and desquamated epithelium in capsular space. Many hyaline casts staining for hemoglobin, many of them containing red blood cells. Glomeruli large. Very marked spontaneous nephritis.
10	$\frac{0.003}{1400}$	0.0021	1	0.1	"	9 "	14.0	Fresh and organized hemorrhages in tufts. Fibrin, serum, red blood cells, and desquamated epithelium in capsular spaces. Half-moons. Very marked leukocytic infiltration of tufts. Frequent epithelial degeneration of tubules. Many hyaline casts.

\* Older, somewhat deteriorated sample of toxin. Doses of 0.007 and 0.01 cc. per kilo failed to cause hemorrhages.

† All doses of bacilli are given in cubic centimeters of a 24 hour broth culture.

TABLE II.

*Controls to Series A. Injection of Colon Bacillus.*

Rabbit No.	Amount of culture injected.	Weight.	Died or killed.	Length of life after injection.	Renal lesions.
		<i>gm.</i>			
11	2 agar slants.	1,500	D.	1½ hrs.	No notable changes.
12	6 cc. of broth culture.	1,500	"	22 "	Considerable excess of leukocytes in tufts. Slight endothelial swelling. No exudate in capsular space.
13	4.5 cc. of broth culture.	1,400	"	36 "	An occasional erythrocyte and desquamated epithelial cell in capsular space. Slight endothelial swelling. A few hyaline casts.
14	3 cc. of broth culture.	1,250	"	5 days.	Moderate endothelial swelling with occasional empty glomerular loops. No exudate.
15	2 cc. of broth culture.	1,450	"	2 hrs.	Marked congestion of glomeruli. No other notable changes.
16	0.1 cc. of broth culture.	1,100	K.	2 days.	No notable changes.

*Series A. An Injection of Diphtheria Toxin Followed by One of Bacillus coli.*

An injection of diphtheria toxin was given 1 to 3 days before the bacteria. The toxin used in this and the next series of experiments was partly from the same sample used in the earlier series in which the effect of toxin alone was determined, and partly from a sample which, though originally of a strength of 175 minimum lethal doses per cc. for guinea pigs, had deteriorated so much that at the time of these experiments it had less than half the strength of the first, as shown by controls. The colon bacilli were given intravenously in doses usually of 0.1 cc. of a 24 hour broth culture. This dosage represented about one-fifth of the minimum lethal dose. The details of the experiments are given in Table I and of the controls in Table II.

The changes were different quantitatively, and also qualitatively from those due to diphtheria toxin alone. A much greater fibrin production is the most striking difference, and on close study it becomes evident that a true fibrinous exudate has occurred in the capsular space from the visceral layer of Bowman's capsule. Associated with the fibrin are seen in varying numbers red blood cells (not constant) and desquamated epithelial cells, the latter derived from the visceral layer, the parietal layer usually appearing to be intact except in occasional glomeruli, where stratification has taken place and the formation of half moons begun. Within the glomeruli, wedge-shaped and circular masses of fibrin are of frequent occurrence. In rabbits killed before these changes are fully developed, a clear, serous exudate is found in the capsular space, accompanied by varying numbers of red cells, while within the tufts globular hemorrhages are sometimes seen. Leukocytes are found, often in large numbers in the neighborhood of the fibrin masses, usually inside the tuft, but also occasionally in the capsular space. Many hyaline and occasional blood casts are present in all the kidneys.

*Series B. An Injection of Vaughan's Split Protein (Poisonous Part) of the Colon Bacillus<sup>1</sup> Following an Injection of Diphtheria Toxin.*

The results of this procedure are shown in Table III.

The renal changes which occurred in the rabbit (No. 20) which received the largest dose of split protein and reacted most severely will be given in detail.

*Rabbit 20.*—The kidneys are deep red and intensely congested throughout. The cortical surface shows a fine, red mottling and occasional pin-point hemorrhages. On the cut surface the rays are seen to be deeply congested, while the cortical surface between them is grayish and swollen. An occasional pin-point hemorrhage in the cortex is seen. The medulla is also deeply congested. With Van Gieson's stain the glomeruli are found to be very large, some of them herniating into the proximal tubule, and many of them contain globular hemorrhages. A few show large collections of red blood cells in the capsular spaces and in others

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<sup>1</sup> I am indebted to Dr. V. C. Vaughan for a supply of split protein (poisonous part) of colon bacillus which he kindly had prepared for me.

TABLE III.  
Series B. Injection of *Diphtheria Toxin* and of *Colon Bacillus Split Protein*.

Rabbit No.	Dose of toxin Weight	Dose per kilo.	Interval.	Dose of colon bacillus split protein.	Died or killed.	Length of life after second injection.	Combined weight of kidneys.	Renal lesions.
17	$\frac{0.002}{1475}$	0.0014	2	0.07	K.	2 days.	11.0	Glomeruli congested. Occasional red blood cells in capsular space. Blood casts and hyaline casts stained with hemoglobin, mostly in ascending loop of Henle. No leukocytic reaction.
18	$\frac{0.002}{1200}$	0.0017	2	0.07	"	2 "	12.1	Occasional red blood cells in capsular space and tubules. No hemorrhages or exudate. Spontaneous nephritis.
19	$\frac{0.002}{1360}$	0.0015	2	0.08	"	2 "	13.0	A few glomeruli show a hemoglobin-tinted clear exudate in capsular space extending down into tubules. Occasional red blood cells in capsular space. A few small hemorrhages in the tufts. Desquamated epithelium in capsular space.
20	$\frac{0.002}{1425}$	0.0014	2	0.1	"	2 "	15.0	Marked glomerular changes. Abundant exudate. (See protocol.)
21	$\frac{0.002}{1375}$	0.0015	2	0.15	D.	15 min.	11.7	No notable changes.
Controls.*								
22	Weight. gm. 1,230			0.1	K.	17 hrs.		No notable changes in either kidney.
23	1,230			0.1	"	17 "		" " " " "
24	1,400			0.1	"	18 "		" " " " "
25	1,500			0.1	"	18 "		" " " " "
26	1,480			0.1	"	19 "		" " " " "

\* All injections in the controls were into the right renal artery.

merely isolated erythrocytes. In the capsular space of a few glomeruli there is a homogeneous, transparent material which takes the characteristic stain for hemoglobin. This material is more abundant in the proximal tubules and is also found in various other parts of the uriniferous tubules, as hyaline, yellow-staining casts. In several glomeruli there is marked epithelial desquamation. There is no increase of leukocytes either in the tufts or in the capsular space. The epithelium of the tubules, especially of the proximal tubules, is beginning to desquamate and the nuclei in many places stain faintly. There is a slight increase of the cells of the glomerular endothelium. Similar changes, though less intense, were present also in the kidneys of Rabbits 17 and 19, which received smaller doses of split protein. It may be pointed out that they did not occur in Rabbit 21, which died 15 minutes after the inoculation.

A few experiments which gave negative or inconclusive results may be briefly mentioned.

*Rabbit 27.*—Weight 1,450 gm. Received 0.001 gm. of uranium acetate intravenously. 6 days later received 0.5 cc. of a 24 hour broth culture of *B. coli communior*, and died in about 12 hours. The kidneys showed an extreme sero-fibrinous exudate in the capsular spaces of a majority of the glomeruli, and also many large hemorrhages within the tufts, besides the usual tubular degeneration of uranium nephritis.

Four other rabbits similarly treated failed to show these glomerular changes.

Rabbits 28 and 29, weighing respectively 1,600 and 1,500 gm., received 0.001 gm. of uranium acetate intravenously and after an interval of 4 days received 0.5 cc. of a 24 hour broth culture of *Streptococcus viridans*. No glomerular lesions were found.

#### DISCUSSION.

Compared with the renal lesions following diphtheria toxin alone, those following successive inoculations of diphtheria toxin and colon bacilli show marked differences. The latter are characterized by a striking tendency to exudation, in the earlier stages, of a clear serous fluid containing small amounts of fibrin and varying amounts, often large, sometimes small, of red blood cells in the capsular space, hemorrhages into the tufts after doses of toxin which alone would not cause hemorrhage, and a fairly constant leukocytic infiltration of the glomeruli. Hyaline casts sometimes containing red blood

cells and occasionally taking the yellow stain of hemoglobin are of frequent occurrence. At a later stage large masses of fibrin in crescents and rings are found on the visceral layer of Bowman's capsule (Figs. 1, 4, 5, and 6), associated with desquamated epithelial cells, occasional erythrocytes and leukocytes, the last named being, however, infrequent in this location. The peripheral layer of the capsule shows in places beginning stratification forming incipient half moons (Figs. 2 and 3). Within the tufts, wedge-shaped or round masses of fibrin are seen which are commonly surrounded by a zone of leukocytic infiltration (Fig. 2).

The changes found after successive injections of diphtheria toxin and Vaughan's split protein of the colon bacillus (poisonous part) also show a marked tendency to exudation (Fig. 7). The capsular spaces of a majority of the glomeruli, when a sufficient amount of split protein has been given, contain an abundant serous fluid stained with hemoglobin which can be traced down the tubules as casts. Red blood cells are sometimes found in the capsular spaces; in the tubules they are frequently embodied in the casts. Intraglomerular hemorrhages occur, though not so commonly as after injections of colon bacilli. A leukocytic reaction was not observed. The later stages of reaction to this type of injury were not studied.

On the whole, the correspondence between the early renal lesions of the two series is close. The main point of dissimilarity, the leukocytic reaction occurring in the first series and absent in the second, admits of explanation as phagocytosis provoked by solid bacterial particles but not by bacterial substance in solution. The appearance of laked hemoglobin in the exudate was more marked in the second series, but was observed occasionally also in Rabbit 3 of the first series.

It seems likely from a study of the two sets of experiments that the lesions in both were essentially of the same nature and it is believed that the mechanism in both cases was the same. The effect of the colon bacillus, in other words, was due to the poisonous molecular groups liberated from its protein by the lytic activities of the body. One must, however, be cautious in making a wide application of this conclusion since in control animals it was not found possible to produce marked renal lesions with repeated injections of colon bacilli



alone or with large amounts of split protein injected directly into the renal artery. Preliminary injury seems to be requisite.

It is interesting to find that the renal lesions of the present experiments correspond closely with those described in man by Volhard and Fahr as the "extracapillary form of glomerulonephritis." The same exudate of fibrin and cells in the capsular space, the same masses of fibrin within the tuft, and the same stratification of the capsular epithelium occur in both.

Without fully agreeing with Ophüls that a distinction between these two forms of glomerulonephritis is "an unnecessary refinement" it seems probable that the extracapillary form reflects a later and severer grade of renal irritation with, perhaps, the addition of a specific reaction of the epithelial elements of the tuft, and from the present experiments it would appear that infection, acting through the poisonous groups split from the protein molecules of bacteria by some lytic reaction in the hosts, is capable of intensifying what was originally a minor grade of injury and thus of producing the severer lesions characteristic of the extraglomerular type.

#### SUMMARY.

1. Rabbits in which a mild grade of glomerular damage has been produced by small doses of diphtheria toxin develop a severe glomerulonephritis corresponding to the extracapillary form described by Volhard and Fahr after inoculations with sublethal quantities of *Bacillus coli*.

2. Similar, though not completely identical lesions are produced when diphtheria toxin is followed by intravenous injections of Vaughan's split protein (poisonous part) of *Bacillus coli*.

3. The inference is drawn that under the experimental conditions described *Bacillus coli* undergoes bacteriolysis and produces glomerular lesions through the toxic groups contained within its protein molecule.

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- Faber, H. K., Studies in glomerulonephritis. I. A quantitative study of the reaction of the kidney to diphtheria toxin, *J. Exp. Med.*, 1917, xxvi, 139.  
Ophüls, W., Nephritis. A new series of cases with a review of recent literature, *J. Am. Med. Assn.*, 1915, lxxv, 1719.  
Volhard, F., and Fahr, T., Die Brightsche Nierenkrankheit, Berlin, 1914. See pp. 33-35.

## EXPLANATION OF PLATES.

## PLATE 11.

FIG. 1. Rabbit 8. Leitz obj. 3. Dosage 0.0013 cc. of toxin per kilo followed by 0.01 cc. of colon bacillus broth. Acute glomerulonephritis. The capsular spaces are filled with fibrin and the tufts are compressed.

FIG. 2. Rabbit 8. Leitz obj. 6. A wedge-shaped fibrin mass, *a*, in the upper glomerulus is surrounded by infiltrated leukocytes, *b*. In the lower glomerulus the capsular epithelium is stratified forming a half moon, *c*.

FIG. 3. Rabbit 8. Leitz obj. 6. Leukocytic infiltration of the glomerulus, *a*, and stratification of the parietal epithelium of the capsule, *b*, are shown.

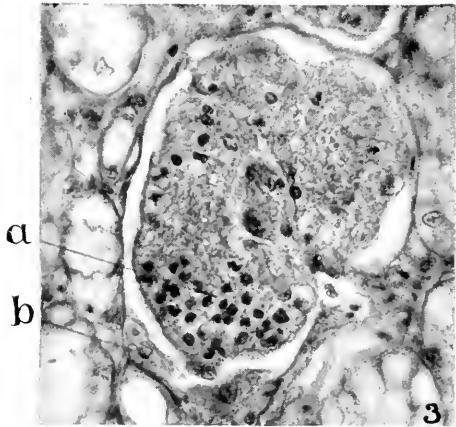
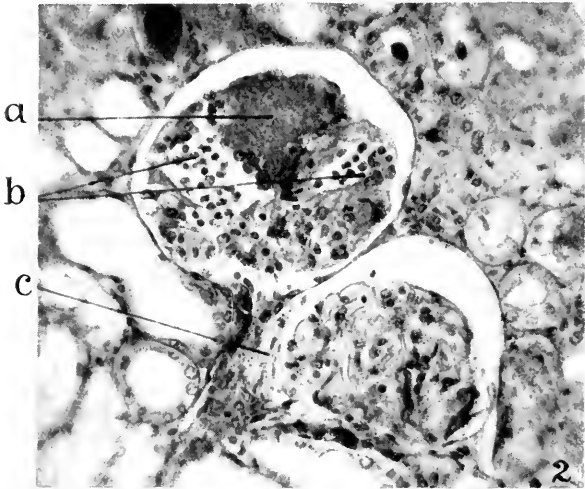
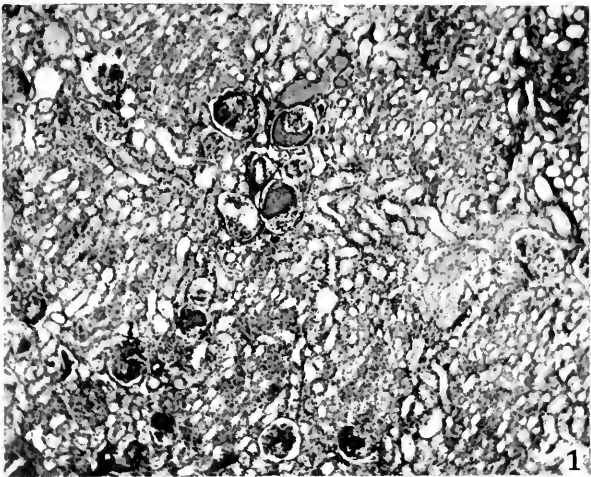
## PLATE 12.

FIG. 4. Rabbit 10. Leitz obj. 6. Dosage 0.0021 cc. of toxin per kilo followed by 0.1 cc. of colon bacillus broth. Enormous dilation of the capsular space, which contains an albuminous exudate, a crescent of fibrin, some desquamated epithelium, and the remnants of the glomerulus, is shown.

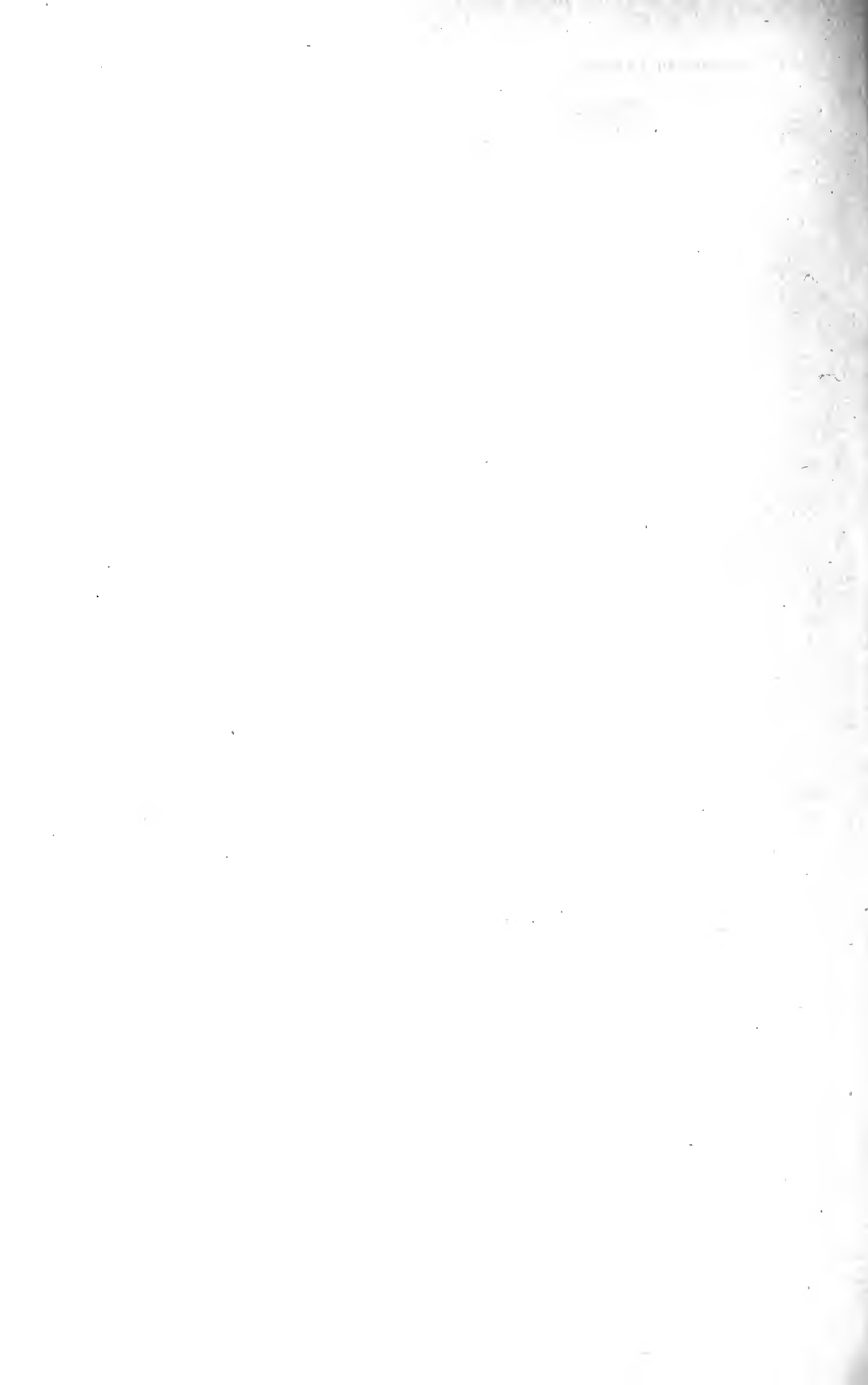
FIG. 5. Rabbit 2. Leitz obj. 6. Dosage 0.0033 cc. of toxin (old) followed by 0.1 cc. of colon bacillus broth. The glomerulus is entirely surrounded by a ring of fibrin. The capsular space is greatly dilated but empty. The capsular epithelium is peeling.

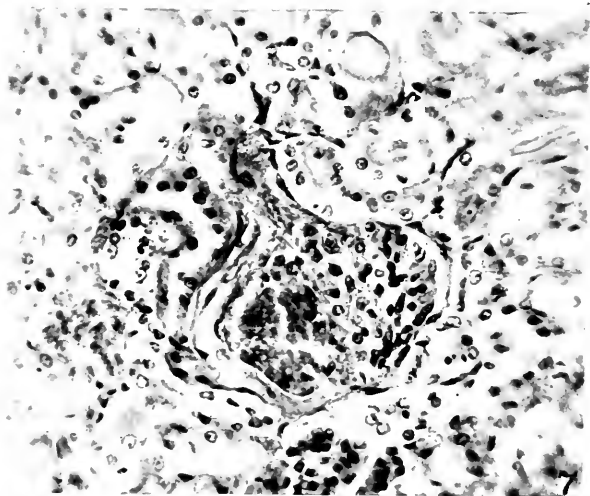
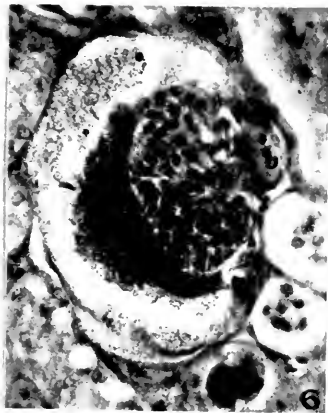
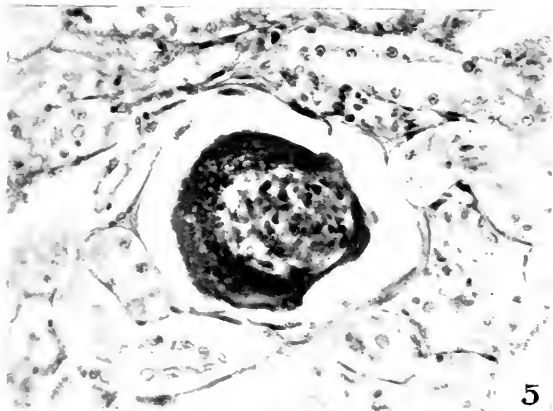
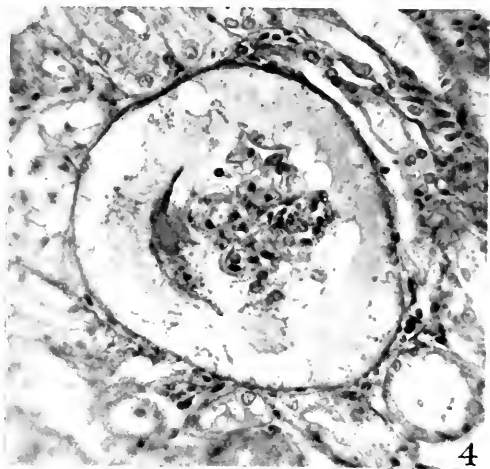
FIG. 6. Rabbit 2. Leitz obj. 6. The glomerulus has a thick cap of fibrin outside of which in the dilated capsular space are masses of red blood cells. The parietal layer of the capsule is beginning to separate.

FIG. 7. Rabbit 20. Diphtheria toxin followed by 0.1 gm. of split protein of the colon bacillus. The glomerulus contains a fresh hemorrhage and the capsular space is filled with a transparent serous exudate which, mixed with granular debris, extends into the neck.



(Faber: Studies in glomerulonephritis. II.)







## EXPERIMENTAL STUDIES UPON LYMPHOCYTES.

### II. THE ACTION OF IMMUNE SERA UPON LYMPHOCYTES AND SMALL THYMUS CELLS.

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PLATE 13.

(Received for publication, February 15, 1917.)

In a recent paper<sup>1</sup> brief reference was made to the production of cytotoxic sera for lymphocytes derived from human tonsils and from the rat thymus. It is desired to report here upon further experiments which have been carried out with these sera, and which appear to bear directly upon the general problem of the specificity of cytotoxins, and upon the important question of the biological identity of the small thymus cells with the lymphocytes found in the lymphoid tissues and in the circulating blood.

The method used in these studies has been described in detail in a previous paper. Briefly stated, it consists in subjecting suspensions of thymus or tonsil cells in salt solution or Locke's fluid for a given period, to the action of whatever toxic agent is chosen, and then adding trypan blue in appropriate dilution. The percentage of diffusely stained cells affords a quantitative measure of the injury produced, when compared with a control suspension maintained under similar conditions. The factors of error, and the precautions necessary in order to make the determinations of value are related in the previous paper.

Brief reference should be made to the more important studies of previous workers, using other methods.

Metchnikoff<sup>2</sup> in 1899 induced leukotoxic sera by injecting spleen and lymph node emulsions of rat and guinea pig into rabbits. He observed the agglutinative

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<sup>1</sup> Pappenheimer, A. M., The reactions of lymphocytes under various experimental conditions, *J. Exp. Med.*, 1917, xxv, 635.

<sup>2</sup> Metchnikoff, E., Études sur la résorption des cellules, *Ann. Inst. Pasteur*, 1899, xiii, 737.

and lytic changes which followed when suspensions were exposed to the action of the immune serum, and described the hydropic swelling of the mononuclears "by which they become transformed into transparent vesicles, the nucleus thereby being rendered very visible." He found that his sera were toxic only for the cells of the species used as antigen, but that there was no evidence of specificity for any particular type of leukocyte, polymorphonuclears and mononuclears being equally affected by anti-lymph-gland serum.

Besredka<sup>3</sup> continuing these studies found that the toxic properties of the leukotoxic sera were destroyed by heating to 55°C. for 30 minutes, and that emulsions of cells heated to 60°C. lost their antigenic properties. He also observed that his sera were mildly hemolytic, although the injected suspensions were macroscopically blood-free. Injections of leukotoxic sera into normal animals produced toxic, or, in large doses, lethal effects. The blood showed an initial hypo-leukocytosis followed by hyperleukocytosis.

Flexner<sup>4</sup> has contributed to the subject a detailed study of the lesions produced in the hematopoietic tissues by myelotoxic and lymphotoxic sera. In the lymph glands the principal changes produced were hyperplasia of the follicles, swelling of the germinal centers, and degeneration of the large cells in the center of the follicles. The degenerative changes in general, however, are described as "minimal and trifling."

Bunting,<sup>5</sup> continuing the work of Flexner, immunized geese with lymph glands and bone marrow of the rabbit, and obtained sera which were "to a large extent specific both in their action on the tonsils and on the circulating blood." One of the sera of the two geese used as controls, however, showed well marked hemolytic powers, and both were agglutinative and lytic for suspensions of lymph gland cells *in vitro*. This action was, however, less intense than that of the serum from a goose immunized against lymph glands.

Christian and Leen<sup>6</sup> have used the cessation of motion of leukocytes observed in a warm chamber as an index of toxicity. They found that sera having both hemolytic and leukotoxic properties could be produced by immunizing with a variety of somatic cells, as those of the liver, spleen, kidney, and cardiac muscle.

The production of thymotoxic sera has been attempted by Moorhead<sup>7</sup> and by

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<sup>3</sup> Besredka, La leucotoxine et son action sur le système leucocytaire, *Ann. Inst. Pasteur*, 1900, xiv, 390.

<sup>4</sup> Flexner, S., The pathology of lymphotoxic and myelotoxic intoxication, *Univ. Penn. Med. Bull.*, 1902, xv, 287.

<sup>5</sup> Bunting, C. H., The effects of lymphotoxins and myelotoxins on the leukocytes of the blood and on the blood-forming organs, *Univ. Penn. Med. Bull.*, 1903-04, xvi, 200.

<sup>6</sup> Christian, H. A., and Leen, J. F., Some further observations on leucocytotoxins, *Boston Med. and Surg. J.*, 1905, clii, 397.

<sup>7</sup> Moorhead, T. G., The thymus gland, *Practitioner*, 1905, lxxv, 733.



Ritchie.<sup>8</sup> Moorhead states briefly that sera from rabbits immunized with guinea pig thymus did not agglutinate thymus cells *in vitro*, and had no leukolytic action. Ritchie, however, using complement fixation methods found that the sera of ducks immunized with suspensions of guinea pig thymus contained an immune body having an affinity for the receptors of the guinea pig thymus and lymph glands, spleen, and bone marrow. In the presence of this immune body, guinea pig complement became fixed, so that there was no hemolysis in the hemolytic system used as a test. The sera showed no affinity for the receptors of the liver, adrenal, thyroid, lung, etc. The serum was not hemolytic, and its binding powers were not affected by heating.

The cytotoxic sera were prepared by injecting rabbits intravenously with washed suspensions of rat thymus cells or of tonsil lymphocytes. The former could be obtained almost blood-free by exsanguinating the rat, carefully dissecting off the superficial blood vessels from the gland, and washing the suspended cells in one or more changes of salt solution. The tonsil suspensions were usually more or less admixed with red blood cells.

#### EXPERIMENTAL.

The following experiments illustrate the cytotoxic and cytoagglutinative properties of these sera, when tested against the appropriate antigen.

*Experiment 1.*—Dec. 5, 1916. Serum C (normal control), Serum H (immunized against human tonsil lymphocytes), and Serum R (from rabbit immunized against rat thymus cells) were added in the proportion of 1:25 to a suspension of rat thymus cells. The tubes were then placed in the incubator for 35 minutes, trypan blue was added in 1:5,000 dilution, and the percentage of stained cells determined. Duplicate counts were made.

Mixture.		Stained cells.	Unstained cells.	Percentage of stained cells.	Agglutination.
Serum C,	0.02 cc.....	195	417	31.8	0
Thymus cells,	0.5 ".....	212	469	31.1	
Serum H,	0.02 ".....	228	208	52.3	0
Thymus cells,	0.5 ".....	322	329	49.5	
Serum R,	0.02 ".....	309	243	55.9	++
Thymus cells,	0.5 ".....	284	194	59.4	++

<sup>8</sup> Ritchie, W. T., The specificity and potency of adrenolytic and thymolytic sera, *J. Path. and Bacteriol.*, 1908, xii, 140.

Although the highest count is obtained with Immune Serum R all the tubes show a high proportion of stained cells. Agglutination occurred only in Serum R.

The sera were then diluted 1:10 and inactivated for 30 minutes at 58°C. A fresh suspension of thymus cells from a healthy young rat was prepared and the following tubes were set up and incubated for 15 minutes at 37°C.

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.	Agglutination.
Serum C, 0.5 cc.....	17	473	3.4	0
Thymus cells, 0.2 ".....				
Serum H, 0.5 ".....	38	433	8.0	0
Thymus cells, 0.2 ".....				
Serum R, 0.5 ".....	27	438	5.8	++
Thymus cells, 0.2 ".....				

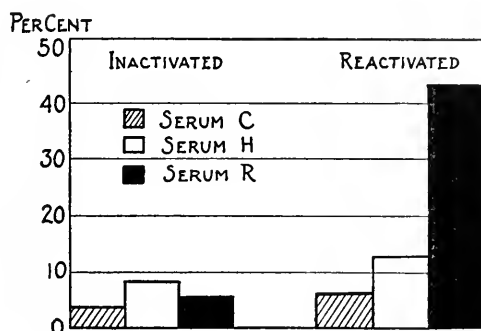
The toxicity of the serum is thus destroyed by heating. Agglutination by Serum R is still marked.

To each tube was added 0.5 cc. of fresh 1:10 guinea pig complement and the tubes were replaced in the thermostat. Counts were made after 1 hour and 15 minutes.

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.	Agglutination.
Serum C, 0.5 cc.....	30	515	5.5	0
Thymus cells, 0.2 ".....				
Complement, 0.5 ".....				
Serum H, 0.5 ".....	60	427	12.3	0
Thymus cells, 0.2 ".....				
Complement, 0.5 ".....				
Serum R, 0.5 ".....	113	150	42.9	+++
Thymus cells, 0.2 ".....				
Complement, 0.5 ".....				

Under the influence of the immune serum the thymus cells are markedly agglutinated, so that there are comparatively few free cells. They are highly irregular in shape, and a great proportion of them are diffusely stained with trypan. Some of the cells show a hydropic swelling of the cytoplasm, the stained nucleus appearing suspended in a clear vesicle. The control sera produced no such effect. The results are shown graphically in Text-fig. 1.

Numerous experiments have given uniform results. Serum R was last tested on January 30, 1917, 26 days after the rabbit had received an injection of thymus cells. The toxicity and agglutinative power were even more marked than in the experiment cited.



TEXT-FIG. 1. Experiment 1. The effect of Thymotoxic Serum R upon the stainability of rat thymus cells.

The following protocol illustrates a similar action of the serum from Rabbit H, immunized with a suspension of human tonsil lymphocytes, when tested against the appropriate antigen.

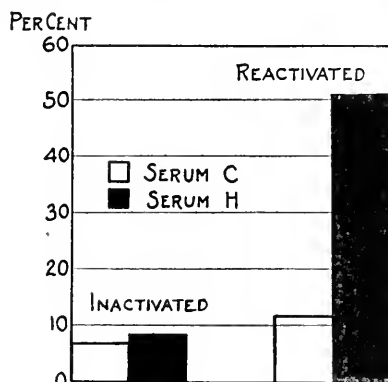
*Experiment 2.*—Dec. 12, 1916. Serum from Rabbit H, which had received four intraperitoneal and two intravenous injections of human tonsil lymphocytes, and Serum C from a control normal rabbit were tested against suspensions of human lymphocytes. The suspension, twice washed and centrifuged, had stood over night in the ice box. Sera were inactivated for 30 minutes at 58°C. and diluted to 1:10.

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.	Agglutination.
Serum H (1:10), 1.0 cc.....	26	283	8.4	+
Tonsil lymphocytes, 0.2 ".....				
Serum C (1:10), 1.0 ".....	27	363	6.9	0
Tonsil lymphocytes, 0.2 ".....				

0.5 cc. of a 1:10 dilution of fresh guinea pig complement was added and the tubes were replaced in the thermostat. Counts made after 45 minutes showed the following:

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.	Agglutination.
Serum H (1:10), 1.0 cc.....	163	156	51.0	++
Tonsil lymphocytes, 0.2 ".....				
Serum C (1:10), 1.0 ".....	40	273	12.7	0
Tonsil lymphocytes, 0.2 ".....				

There is thus a striking increase in the proportion of stained cells under the influence of the immune serum, accompanied by macroscopic agglutination, and the morphological changes described above. The results are shown in Text-fig. 2.



TEXT-FIG. 2. Experiment 2. The effect of lymphocytotoxic serum upon the stainability of human tonsil lymphocytes.

Repeated observations having assured us of the constancy of this cytotoxic action of Anti-thymus Serum R, the effects produced *in vitro* were compared with those which might be caused by injection into the living animal.

*Experiment 3.*—Dec. 14, 1916. Two young rats were etherized and a fragment of thymus was removed from each for histological control. Sections showed a normal structure.

Dec. 19. Rat A was injected intraperitoneally with 1 cc. of native Anti-thymus Serum R. The control, Rat B, received normal rat serum. After 24 hours the rats were killed and suspensions in Locke's fluid were made from a portion of each thymus gland, the remainder being fixed and sectioned. Counts of the suspensions, with and without the addition of rat serum, showed no significant

Experiment 4, however, in which the sera were first inactivated and complement was injected simultaneously gave definite indication of cytotoxic action *in vivo*, and also confirms the validity of the method used in determining cell injury.

*Experiment 4.*—Dec. 21, 1916. Rats A and B, well nourished young animals of approximately the same age and weight, were etherized and fragments of thymus excised from each. Portions of the fragments were fixed in Zenker's fluid, sectioned, and found to show a normal structure. The remainder was teased in Locke's solution and counts were made in the usual manner.

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.
Rat A thymus cells in Locke's fluid.....	30	551	5.1
" B " " " " " " .....	44	442	9.0

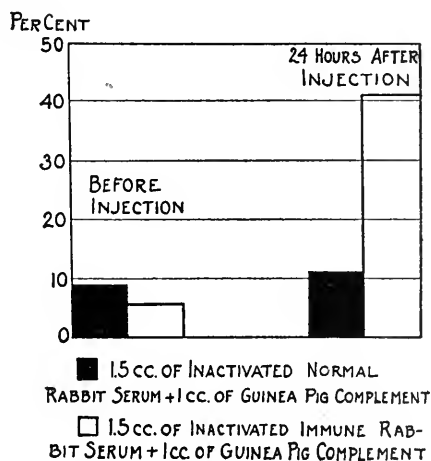
Rats killed after 24 hours. The thymus of each was divided into two parts; one was used for determining the stainability of cells, the other fixed and sectioned. The count shows the following, after 1 hour at 37°C.

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.
Rat A thymus cells in 0.85 per cent salt solution.....	112	156	41.7
Rat B thymus cells in 0.85 per cent salt solution.....	58	424	12.0

The study of the histological preparations indicates that the thymus of Rat A has been severely damaged by the cytotoxic serum

(Fig. 1). The gland which macroscopically was smaller and firmer than the control shows a great rarefaction of the cortex, fragmentation of the small thymus cells, and clumps of bluish staining material (remains of altered chromatin) in and amongst the conspicuous reticular cells. The interlobular septa are edematous and contain wandering cells of different types. There are no hemorrhages.

There is thus a convincing correspondence between the lesions produced and the increased fragility of the cells under the influence of the immune serum *in vitro*. The negative results of the previous experiments, in which the native serum was injected, are not ex-



TEXT-FIG. 3. Experiment 4. The stainability of rat thymus cells before and after the injection of immune serum.

plained. Later repetitions of this experiment have not been uniformly successful. In some instances we have observed a pronounced destruction of small thymus cells with phagocytosis of the reticular epithelium; in other cases no effects were produced. It is evident that there are variable factors which have not yet been determined. It is perhaps in accord with the well known phenomenon that, as Zinsser<sup>9</sup> states, "the alexin of an animal is entirely impotent or but weakly capable of producing hemolysis of the sensitized cells of its own species."

<sup>9</sup> Zinsser, H., *Infection and resistance*, New York, 1914, 154.

This possible explanation, however, was tested by comparing the complementary action of guinea pig serum and rat serum, in the presence of anti-thymus cell amboceptor and washed thymus cells. It was found that whereas 0.2 cc. of guinea pig serum was sufficient to cause a maximal cytotoxic effect in the presence of immune serum (0.5 cc.) and thymus cell suspension 0.2 cc., 0.4 cc. or double the amount of rat serum was required. Whether this slight difference in the complementary action, which was determined in only a single experiment, is a constant one, cannot be stated. It hardly affords a sufficient explanation for the negative experiment, and further studies are necessary to clear up this point.

Previous workers with leukotoxic and lymphotoxic sera have invariably found a moderate hemolytic activity upon the red cells of the species furnishing the antigen. The sera studied by us have also been moderately hemolytic, as shown by the following protocol.

*Experiment 5.*—Serum H from a rabbit which had been injected with human tonsil lymphocytes was tested against washed human erythrocytes.

Serum H (1 : 10) inactivated.	5 per cent red blood cells.	Salt solution.	10 per cent guinea pig com- plement.	Hemolysis.	Agglutination.
cc.	cc.				
1.0	0.5	0.5	0.5	++	+
0.5	0.5	1.0	0.5	+	0
0.1	0.5	1.4	0.5	0	0
0.05	0.5	1.45	0.5	0	0
0.01	0.5	1.49	0.5	0	0
0.00	0.5	2.00	0.5	0	0

Readings after 1 hour at 37°C. and 18 hours in the ice box showed that the serum caused slight hemolysis in a dilution of 1:50. The thymotoxic serum (R) was also feebly hemolytic for rat corpuscles.

Jan. 29, 1917.

*Experiment 6.*

Serum R (1 : 10) inactivated.	5 per cent red blood cells.	Salt solution.	10 per cent guinea pig com- plement.	Hemolysis.	Agglutination.
cc.	cc.				
1.0	0.25	0.75	0.5	+	0
0.5	0.25	1.25	0.5	+	0
0.1	0.25	1.65	0.5	0	0
0.0	0.25	1.75	0.5	0	0

This brings up at once the question as to whether the thymotoxic and thymoagglutinative factors are distinct from the hemolytic ones. That the sera should be mildly hemolytic was to be expected not only from the concurrent experience of previous workers, but from the fact that the cell suspensions could not be rendered wholly blood-free even by repeated washing.

We have attacked the problem in two ways: first, by determining whether, after complete absorption of the hemolysin, the serum still retained to a degree its thymotoxic and thymoagglutinative properties; and secondly, by studying the effects upon the thymus cells of a serum prepared by immunizing a rabbit against rat erythrocytes.

Experiment 7 illustrates the fact that both thymotoxic and thymoagglutinative factors persist after complete absorption of the hemolysin and hemagglutinin.

*Experiment 7.*—Jan. 29, 1917. Anti-thymus Serum R from the rabbit whose last injection with thymus cells had been on Jan. 5 was inactivated. The hemolytic activity of the serum was first tested, and it was found that 0.1 cc. of serum in the presence of 0.5 cc. of 10 per cent complement completely hemolyzed 0.25 cc. of washed rat erythrocytes.

A preliminary test was also made of its cytotoxic property for thymus cells. The percentage of stained cells rose after reactivation of the serum from 6.2 to 100 per cent and agglutination was marked.

To absorb out the hemolytic factor 2 cc. of 5 per cent red cells were added to 2 cc. of inactivated serum. After 1 hour at 37°C. the red cells were removed by centrifugation, and 2 cc. of red blood cells were again added. The erythrocytes were then allowed to remain in contact with the serum in the ice box for 18 hours. On the following day the serum was pipetted off and tested both for its hemolytic activity and for its thymotoxic action.

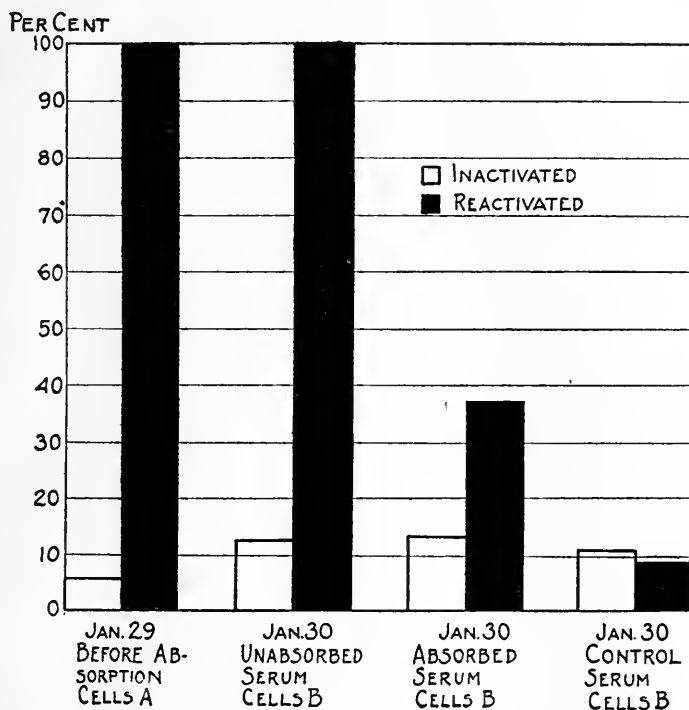
0.1 cc. of the absorbed serum now completely failed to hemolyze 0.25 cc. of washed erythrocytes in the presence of 0.5 cc. of complement. A definite though diminished toxicity for the thymus cells could still be demonstrated, the percentage of stained cells after reactivation rising from 12.8 to 37.0 per cent. A control normal serum, tested against the same suspension, showed no increase in the percentage of stained cells, after addition of complement. These relations are shown graphically in Text-fig. 4.

Several other experiments conducted in the same manner have given comparable results. One may conclude from them that either (a) there is a toxic factor distinct from the hemolytic one or (b) the hemolytic factor persists in the absorbed serum in minimal



amount, which is impotent to cause laking of the erythrocytes, but is still sufficient so to alter the permeability of the lymphocytes that the percentage of stained cells is increased. There seems at present no way of deciding which of these two suppositions is the correct one.

In the hope of obtaining further information upon the question of the identity of hemolytic and thymotoxic substances, a hemolytic serum was prepared by injecting a rabbit with washed rat erythro-



TEXT-FIG. 4. Experiment 7. Persistence of thymotoxins and thymoagglutinins after the absorption of hemolysin from thymotoxic serum.

cytes. The toxicity of this serum for thymus cells was then tested, and the separation of the two factors by absorption with red cells and thymus cells, respectively, attempted.

*Experiment 8.*—Jan. 23, 1917. Serum from Rabbit H which had received four spaced injections of washed rat corpuscles intravenously was obtained 12 days after the last injection.

*A. Preliminary Hemolytic Titration.*

Serum (1:10) inactivated.	Red blood cells.	Complement	Hemolysis.	Agglutination.
cc.	cc.			
1.0	0.25	0.5	+	+++
0.5	0.25	0.5	+	+++
0.25	0.25	0.5	+	++
0.12	0.25	0.5	+	+
0.0	0.25	0.5	0	0
1.0	0.25		0	0

The serum is moderately hemolytic in dilution up to 1:400 although the hemolysis is masked somewhat by the strong agglutination.

*B. Preliminary Test for Thymotoxic and Thymoagglutinating Properties.*

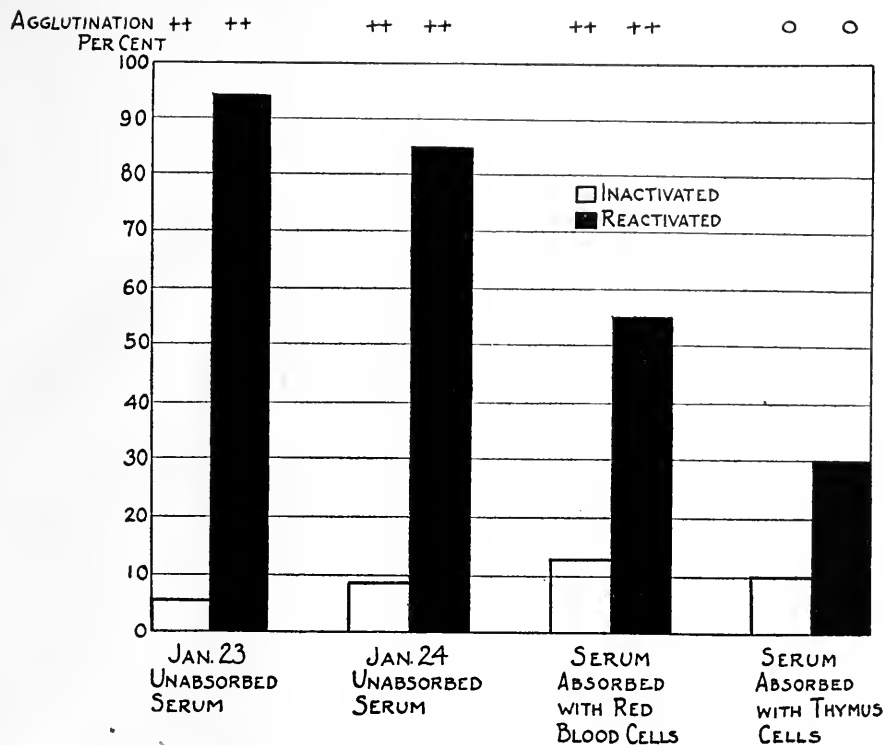
Mixture.	20 min. in incubator.			30 min. after the addition of 0.5 cc. of 10 per cent guinea pig complement.
	Stained cells.	Unstained cells.	Percentage of stained cells.	Percentage of stained cells.
Serum (1:10) inactivated, 0.05 cc. Thymus cells, 0.1 "	27	458	5.5	94.5

*C. Absorption of Hemolysin and Hemagglutinin.*

Inactivated serum (1:10).....3 cc.  
5 per cent rat erythrocytes .....3 "

In the incubator 1 hour at 37°C. Centrifuged serum was pipetted off and 1 cc. of fresh suspension added. Placed in the ice box over night. The following morning the completeness of the absorption was tested. It was found that 0.1 cc. produced neither hemolysis nor agglutination of 0.25 cc. of red corpuscles in the presence of 0.5 cc. of 10 per cent complement. The absorption of the hemolysis was, therefore, apparently complete.

The toxicity of the absorbed and unabsorbed sera for rat thymus cells was then compared, a fresh suspension from a healthy young animal being taken (Text-fig. 5).



TEXT-FIG. 5. Experiment 8. Persistence of the thymotoxic factor in hemolytic serum after absorption.

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.	Agglutination.
Absorbed serum (1:10) inactivated, 1.0 cc. Thymus cells, 0.1 cc.	40 min. in incubator.			
	73	456	13.8	— —
	45 min. after the addition of 0.5 cc. of 10 per cent guinea pig complement.			
	315	251	55.7	
Unabsorbed serum (1:10) inactivated, 1.0 cc. Thymus cells, 0.1 cc.	40 min. in incubator.			
	50	457	9.8	++
	0.5 cc. of 10 per cent guinea pig complement added. 45 min. in incubator.			
	388	67	85.3	

The serum, after apparently complete absorption of hemolysin and hemagglutinin, is still strongly agglutinative and toxic for thymus cells.

To determine whether any of the agglutinin for thymus cells had been removed by exposure of the serum to red blood corpuscles a rough quantitative comparison of the agglutinin present in the absorbed and unabsorbed serum was made.

Unabsorbed serum (1:10).			Absorbed serum (1:10).		
Serum.	Salt solution.	Agglutination.	Serum.	Salt solution.	Agglutination.
<i>gll.</i>	<i>gll.</i>		<i>gll.</i>	<i>gll.</i>	
10	0	+++	10	0	+++
8	2	++	8	2	+++
6	4	++	6	4	++
3	7	=	3	7	++
1	9	0	1	9	0
0	10	0			

1 drop of a thick suspension of thymus cells to each tube was used. Macroscopic readings were made after 1 hour at 37°C. There appears to be no quantitative diminution in the amount of agglutinin for thymus cells.

Experiments were undertaken to absorb out the hemagglutinative and hemolytic factors by exposing the serum to suspensions of washed thymus cells. This was done, an equal volume of a thick suspension being added, and after 1 hour in the incubator, the serum was centrifuged and pipetted off and fresh cells were allowed to stand in contact with the serum for 18 hours in the ice box.

The serum was then tested for its hemolytic and hemagglutinative powers. These were found to have been practically unaffected, 0.1 cc. upon the addition of complement still causing complete hemolysis and strong agglutination of 0.25 cc. of rat corpuscles. The thymoagglutinative and thymotoxic factors, though much reduced were still present.

To sum up this experiment, which has been repeated with similar results, one finds that (1) hemolytic serum obtained by immunizing rabbits with washed rat erythrocytes is both toxic and agglutinative for small thymus cells; (2) the thymotoxic and thymoagglutinative factors, however, cannot be completely removed by absorption of the hemolysins and hemagglutinins; (3) the hemolysin and hemagglutinin cannot be absorbed by exposure of the serum to suspensions of thymus cells.

The evidence seems to favor the view that we are dealing with two distinct factors. If we assume that the thymus cells are identical with the lymphocytes of the circulating blood, it seems probable

that specific immune bodies have been produced in response to the lymphocytes injected with the washed erythrocytes. The alternative view is the one already suggested; namely, that minute amounts of hemolysin, insufficient to cause laking of the red cells, are still able to produce the striking cytotoxic effects upon the thymus cells recognizable by the trypan blue method.

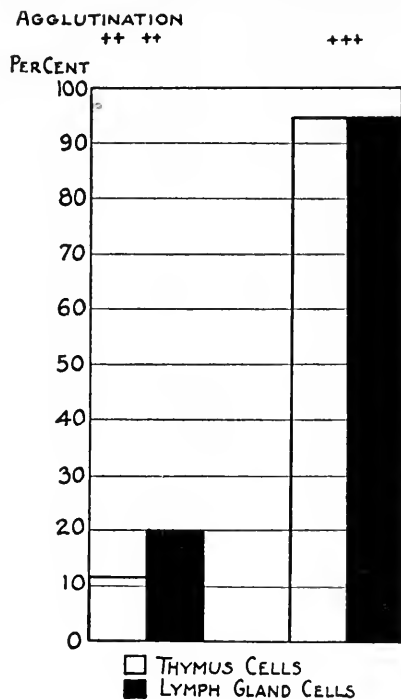
Although we have not been able to work with lymphocytes from the circulating blood, we have studied the action of thymotoxic serum upon suspensions of lymphocytes derived from lymph glands. The following illustrative protocol shows that, as far as their reaction to the immune serum is concerned, thymus cells and lymph gland lymphocytes are identical.

*Experiment 9.*

Jan. 30, 1917.

Mixture.	Stained cells.	Unstained cells.	Percentage of stained cells.	Agglutination.
Anti-thymus serum (1:10), 1.0 cc. Lymph gland lymphocytes, 0.2 "	55 min. in incubator.			
	112	431	20.6	--
	45 min. after the addition of 0.5 cc. of complement.			
	493	27	94.8	
Anti-thymus serum (1:10), 1.0 cc. Thymus cells, 0.2 "	55 min. in incubator.			
	62	477	11.5	--
	1 hr. after the addition of 0.5 cc. of complement.			
	487	25	95.1	

Both lymph gland lymphocytes and small thymus cells are severely injured after exposure to the activated anti-thymus serum (Text-fig. 6). It is not possible to secure from the small lymph nodes of the rat sufficiently large suspensions to make absorption tests satisfactory. The experiment as it stands, however, seems strongly to point to a close biological relationship between the thymus cells and lymphocytes from other sources.



TEXT-FIG. 6. Experiment 9. Comparison of the action of thymotoxic serum upon thymus cells and lymph gland lymphocytes.

#### DISCUSSION AND SUMMARY.

The work of previous investigators gives the impression that it is easy to produce sera which both *in vitro* and upon injection are leukotoxic. At the same time the specificity of these leukotoxic sera for the particular type of cell used as antigen, and even for leukocytes in general, has been doubtful. The methods used have made certain possible factors of error unavoidable. Even careful washing of an organ or suspension cannot render it wholly blood-free, so that it is not surprising that the sera should be moderately hemolytic and hemagglutinative. Pearce<sup>10</sup> has shown that the injection of very

<sup>10</sup> Pearce, R. M., Concerning the specificity of the somatogenic cytotoxins, *J. Med. Research*, 1904, xii, 1.

small amounts of blood is sufficient to evoke the production of immune hemolysins. When such sera are injected the lesions, as Pearce states, may be due in part to the production of hemagglutinative thrombi, although this hardly seems to apply to the changes in lymphoid tissue described by Flexner. On the other hand, the lymphotoxic effect of hemolytic sera may be due to the lymphocytes injected with the red cells.

Our own experiments indicate that the lymphotoxic and agglutinative factors are to a considerable degree distinct from the hemolytic and hemagglutinative ones, since they can be separated from one another by absorption.

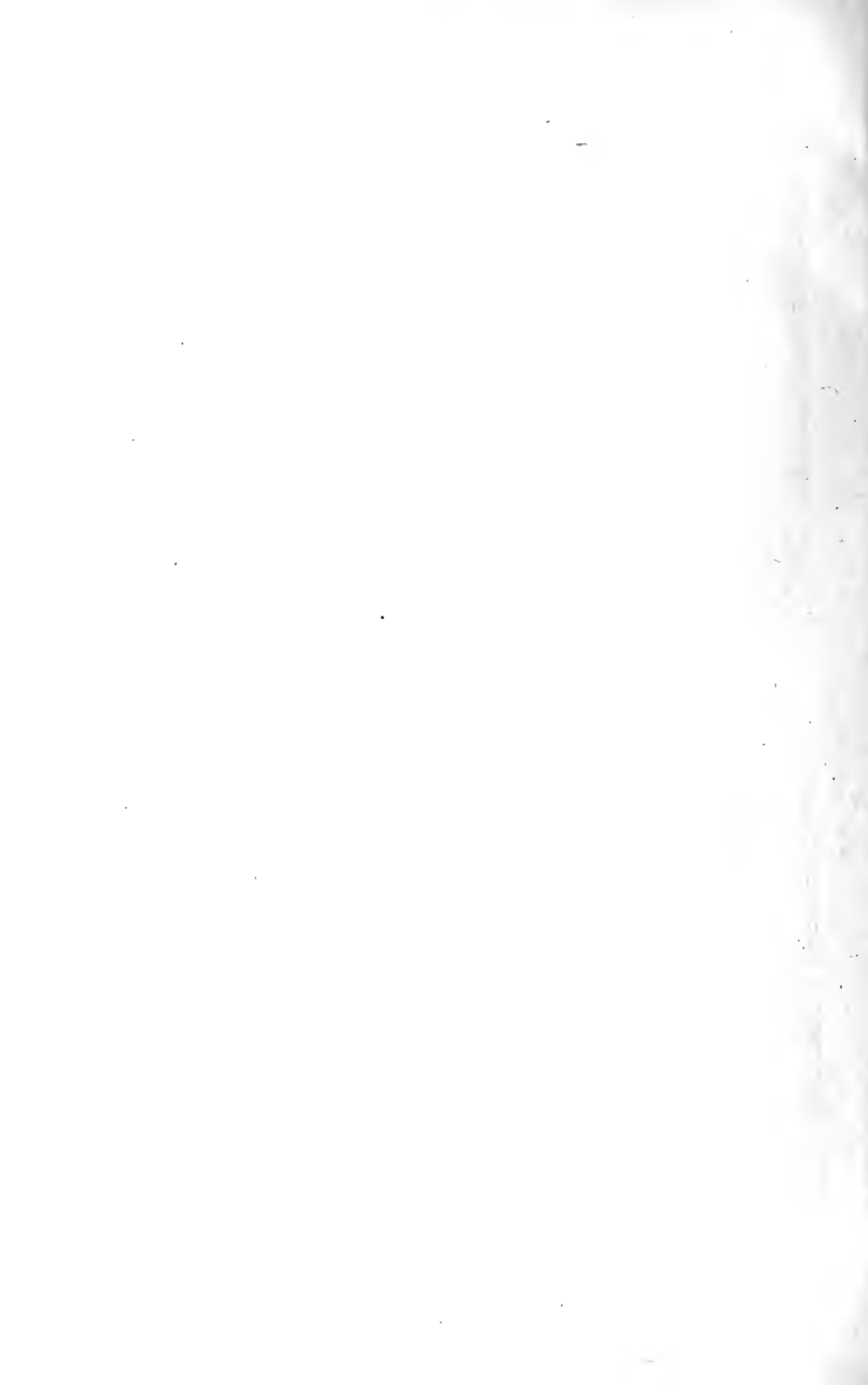
Further evidence is presented that the small thymus cells are biologically related to, if not identical with the lymphocytes derived from lymph glands.

I wish to acknowledge the assistance of Miss Kate Brogan in the technical part of the work.

#### EXPLANATION OF PLATE 13.

FIG. 1. Rat A. Thymus 24 hours after the injection of immune serum and complement.

FIG. 2. Rat B. Thymus 24 hours after the injection of normal serum and complement.





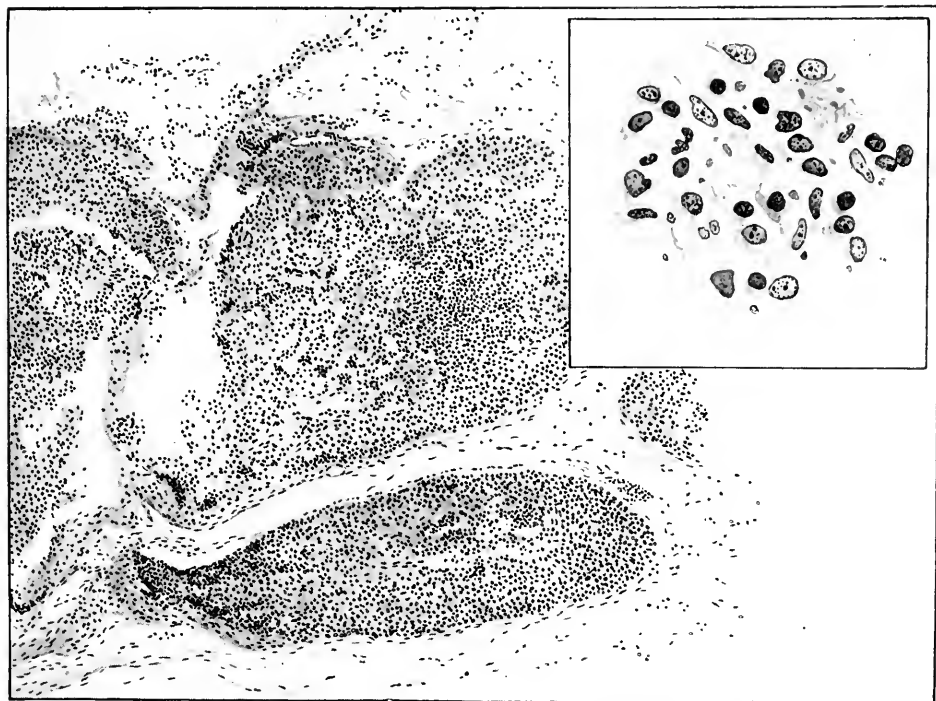


FIG. 1.

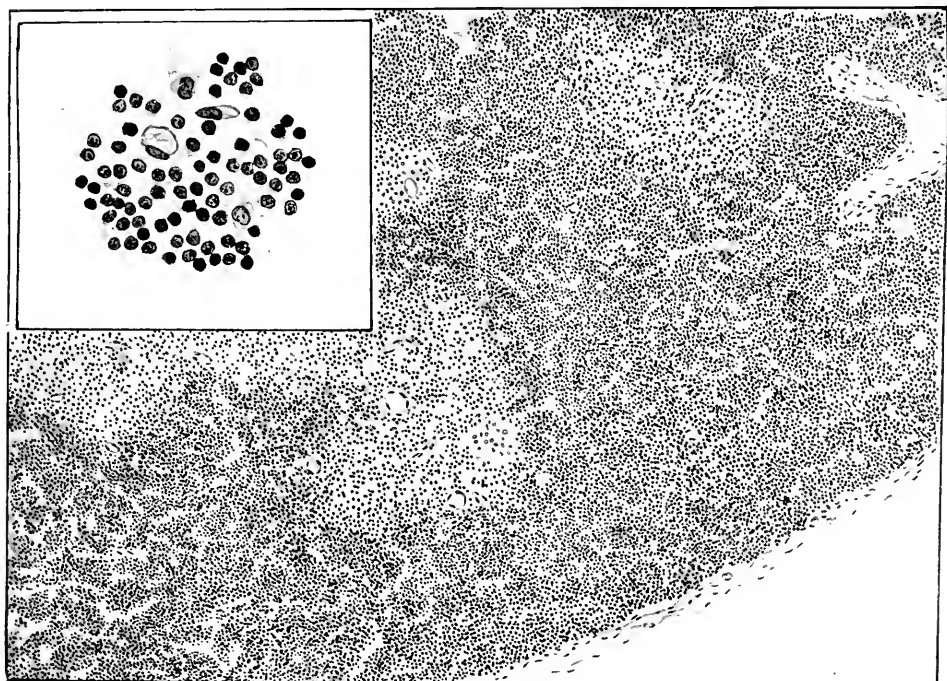


FIG. 2.

(Pappenheimer: Experimental studies upon lymphocytes. II.)



## THE MECHANISM OF UREA RETENTION IN NEPHRITIS.

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The present paper furnishes additional evidence in support of the view of the mechanism of urea retention in Bright's disease advanced by Widal and Javal in 1904 (1). Upon their conception of this mechanism was based the later work of Ambard and Weill (2) and of the author (3). On account of a present general misconception of the mechanism of urea retention, it seems advisable to add further evidence in favor of the hypothesis originated by the French school, although all credit for the conception must be given to these writers. It is not proposed at this time to discuss the respective merits of diagnostic tests of renal function, either in regard to their reliability or simplicity. These studies have been carried on with the view of establishing certain laws which have biological significance and are apparently valid.

For the purposes of the present paper the term urea retention is intended to mean a relatively increased concentration of urea in the blood and tissues, such as may occur over rather long periods of time in acute and chronic nephritis and is attributed to a disturbance in renal function.

It does not refer to the more rapid accumulation of urea which may occur as a result of partial or complete suppression of urine, due not to a specific defect in the function which has to do with the excretion of urea, but to a suspension of that function which has to do with the elimination of fluid through the kidneys. Such accumulation commonly occurs in oliguria due to passive congestion of the kidneys, or to any other cause. Neither does it refer to the long continued positive nitrogen balance which has been described by some authors.

Widal and Javal (1) kept a woman aged 34 years under observation for 4 months, during which time the nitrogen balance was carefully observed. The patient

had been ill for 1 year with chronic nephritis and the general condition remained unchanged during the period of observation. The urine albumin varied between 1.0 and 2.5 gm. per liter. The chloride balance was perfect. They found that the patient came into nitrogen equilibrium readily after changes in the nitrogen intake, though somewhat more slowly than a normal individual. They also found that the concentration of urea in the blood bore a direct relation to the diet, and on a constant nitrogen intake was practically constant. After changes in diet the blood urea could be restored to the former level by a return to the original diet. They succeeded in causing the concentration of urea in the blood to increase from 0.36 to 1.93 gm. per liter by increasing the nitrogen intake. As a result of their experiment, they concluded that the fluctuations in the level of blood urea were due to the changes in diet, and that the increased concentration of urea in the blood, which occurred in response to increased nitrogen intake, effected the elimination of increased amounts of urea through the kidneys. The organism therefore remained in nitrogen equilibrium. In other words, high nitrogen intake, relatively high blood urea, and high rate of excretion were found to be parallel and interdependent phenomena, as were low nitrogen intake, relatively low blood urea, and low nitrogen output. The term relative is used since, as has been repeatedly pointed out, there can be no absolute standard for the concentration of urea in the blood, either in health or in disease. The above hypothesis received further support from the work of Ambard and Weill (2), who demonstrated that the rate of urea excretion depends primarily on the concentration in the blood. Numerous authors have confirmed the observation of Widai and Javal on the dependence of the concentration of urea in the blood on the level of protein intake.

The present paper deals chiefly with the detailed study of two cases similar to the study made by Widai and Javal, with a simultaneous study of the state of the urea excretion function by the index of urea excretion, based on Ambard's laws, previously described (3). These two cases reveal the interrelationship between nitrogen intake, blood urea concentration, and nitrogen output, and show that changes in the separate factors may occur while no change in the actual ability of the kidneys to excrete urea is demonstrable. Additional data are presented, from other cases, which show in common a characteristic not previously described.

#### *Methods of Observation.*

The two patients first described were cases of chronic nephritis selected on account of their diminished urea-eliminating function, with little or no impairment of the function of water excretion. They

were ambulatory patients, apparently not in a progressing state of the disease, and were able to tolerate the dietary changes well. On account of the length of the experiments it was thought advisable to allow a somewhat more varied diet than is usual in metabolism experiments, but the protein-containing foods were analyzed frequently, and were not varied from day to day. Protein was given chiefly in the form of eggs, bread, and milk. The chloride and fluid intake were also kept constant. Urine and stools were collected for 24 hour periods and analyzed daily for nitrogen. Blood urea was determined about twice a week, with a simultaneous urine analysis on a carefully timed specimen, in order to compute the index of urea excretion.<sup>1</sup>

Both the patients tolerated the regimen well and showed little or no change in the general condition at the end of the experiment.

*Case 1.*—C. P., male; age 48 years (Table I, Text-fig. 1).

*Diagnosis.*—General arteriosclerosis, chronic interstitial nephritis.

The patient was first admitted to the hospital on September 14, 1915, and was kept under observation until June 5, 1916. He complained of general weakness and loss of weight which had been progressing for 4 or 5 years. Physical examination revealed an advanced grade of peripheral arteriosclerosis. The heart was slightly enlarged. The rate and rhythm were normal. There were no murmurs. Fluoroscopic examination and the x-ray plate of the chest showed an enlarged aortic arch. The blood pressure was 180 systolic and 110 diastolic. The urine was clear, straw-colored, neutral; specific gravity 1,011. There was a heavy trace of albumin, but no sugar. Hyaline and finely granular casts were present, but not in large numbers. No red blood cells were found. The total elimination of phenolsulfonephthalein was 30 per cent in 2 hours on September 15.

The patient reentered the hospital in October and the nitrogen balance was determined from October 26 to December 29. The findings during this period

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<sup>1</sup> The index of urea excretion is computed from the concentration of urea in the blood, the rate of urea excretion, the concentration of urea in the urine, and the weight of the patient, according to the following formula.

$$\text{Index of urea excretion (I)} = \frac{D \sqrt{C} \times 8.96}{\text{Wt.} \times \text{Ur.}^2}$$

D = gm. of urea excreted per 24 hours.

C = " " " per liter of urine.

Ur. = " " " " " " blood.

Wt. = body weight of individual, in kilos.

For further details consult the description of the index in previous papers (3).

TABLE I.  
Case I.

Date.	Weight.	Diet per 24 hrs.				Urine per 24 hrs.			Daily nitrogen balance.							Urea		Blood pressure.	
		Protein.	Calories.	Sodium chloride.	Fluid.	Sp. gr.	Albumin per liter (Esbach).	Quantity.	Urea N per liter of urine.	Total N per liter of urine.	Total urine N in 24 hrs.	Stool N in 24 hrs.	Total N.			Urea per liter of blood.	Index of excretion (l).	Systolic.	Diastolic.
1915	kg.	gm.		gm.	cc.		gm.	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.			
Oct. 26	66.4	120.0	2,501	10.0	3,000	1.009	0.3	3,537	4.12	4.52	16.0	1.6	17.6	19.2	+1.6	0.825	18.4	190	120
" 27	65.2	120.0	2,501	10.0	3,000	1.010	0.2	2,719	4.25	5.30	14.4	2.0	16.4	19.2	+2.8	—	—	—	—
" 28	66.0	120.0	2,495	10.0	3,000	—	—	3,145	—	—	Lost.	3.0	—	19.2	—	—	—	—	—
" 29	65.2	120.0	2,495	10.0	3,000	1.010	0.25	2,540	5.18	6.25	15.9	3.2	19.1	19.2	+0.1	0.832	17.8	190	120
" 30	65.4	120.0	2,495	10.0	3,000	1.010	Tr.	3,265	4.32	5.04	16.5	2.5	19.0	19.2	+0.2	—	—	—	—
" 31	65.0	120.0	2,506	10.0	3,000	1.010	0.3	2,650	4.96	5.82	15.4	3.3	18.7	19.2	+0.5	—	—	—	—
Nov. 1	65.2	120.0	2,506	10.0	3,000	1.013	0.3	2,420	5.96	6.42	15.5	2.4	17.9	19.2	+1.3	—	—	200	120
" 2	65.4	120.0	2,506	10.0	3,000	1.012	0.3	2,930	4.45	5.15	15.1	2.7	17.8	19.2	+1.4	0.835	19.4	—	—
" 3	65.0	120.0	2,506	10.0	3,000	1.013	0.3	2,773	4.91	5.39	14.9	2.8	17.7	19.2	+1.5	—	—	—	—
" 4	64.8	120.0	2,506	10.0	3,000	1.010	0.25	2,568	4.81	5.79	14.9	3.1	18.0	19.2	+1.2	—	—	—	—
" 5	65.0	120.0	2,509	10.0	3,000	1.011	0.25	2,691	5.44	6.00	16.1	3.1	19.2	19.2	0	0.858	22.0	—	—
" 6	64.6	60.0	2,497	10.0	3,000	1.010	—	2,435	4.12	5.69	13.8	1.1	14.9	9.6	-5.3	—	—	197	110
" 7	64.6	60.0	2,497	10.0	3,000	1.010	0.25	1,931	4.64	5.52	10.7	3.5	14.2	9.6	-4.6	—	—	—	—
" 8	64.6	60.0	2,493	10.0	3,000	1.010	Tr.	2,553	2.99	3.78	9.7	1.0	10.7	9.6	-1.1	—	—	192	110
" 9	64.6	60.0	2,497	10.0	3,000	1.008	0.2	2,270	3.38	4.00	9.1	1.8	10.9	9.6	-1.3	0.569	17.4	200	130
" 10	64.6	60.0	2,497	10.0	3,000	1.007	0.1	2,073	3.16	3.88	8.0	1.1	9.1	9.6	+0.5	—	—	206	130
" 11	64.0	60.0	2,497	10.0	3,000	1.005	0.2	2,272	2.63	3.54	8.0	2.7	10.7	9.6	-1.1	—	—	—	—
" 12	64.8	60.0	2,493	10.0	3,000	1.008	0.2	2,026	3.41	3.91	7.9	1.6	9.5	9.6	+0.1	0.499	23.2	195	125
" 13	65.0	60.0	2,493	10.0	3,000	1.008	0.2	2,171	3.05	3.57	7.7	2.1	9.8	9.6	-0.2	—	—	190	110
" 14	64.8	60.0	2,493	10.0	3,000	1.009	0.25	1,988	3.42	3.82	7.6	1.7	9.3	9.6	+0.3	—	—	—	—

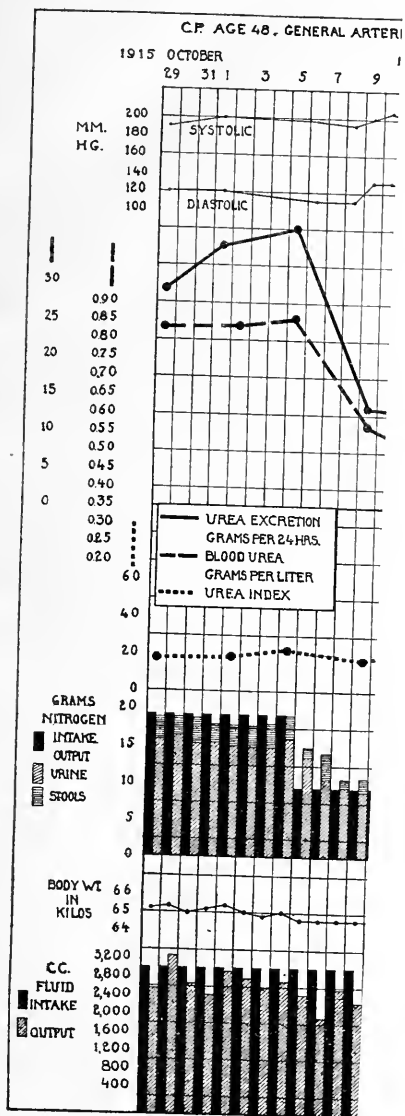
Nov. 15	64.6	60.0	2,497	10.0	3,000	1,007	0.1	1,918	2.75	3.50	6.7	1.5	8.2	9.6	+1.4	—	—	194	110
" 16	64.8	60.0	2,411	10.0	3,000	1,007	0.2	2,426	1.99	2.72	6.6	2.9	9.5	9.6	+0.1	0.411	24.0	—	—
" 17	64.8	60.0	2,411	10.0	3,000	1,006	0.25	2,251	2.66	3.19	7.2	1.9	9.1	9.6	+0.5	—	—	—	—
" 18	65.0	60.0	2,411	10.0	3,000	1,007	0.25	2,141	2.55	3.14	6.7	1.6	8.3	9.6	+1.3	—	—	—	—
" 19	65.4	60.0	2,411	10.0	3,000	—	Tr.	1,996	2.79	3.57	7.1	1.6	8.7	9.6	+0.9	0.402	21.0	—	—
" 20	65.0	30.0	2,798	10.0	3,000	1,006	0.2	1,915	2.26	3.15	6.0	1.1	7.1	4.8	-2.3	—	—	205	120
" 21	65.4	30.0	2,798	10.0	3,000	1,006	0.1	2,073	1.71	2.19	4.5	1.3	5.8	4.8	-1.0	—	—	—	—
" 22	65.0	30.0	2,798	10.0	3,000	1,006	Tr.	1,936	1.50	2.24	4.3	1.2	5.5	4.8	-0.7	—	—	—	—
" 23	64.6	30.0	2,798	10.0	3,000	1,005	0.2	2,177	1.36	1.78	3.9	1.2	5.1	4.8	-0.3	0.269	32.0	195	110
" 24	65.2	30.0	2,798	10.0	3,000	1,008	11.Tr.	1,852	1.27	1.97	3.6	1.6	5.2	4.8	-0.4	—	—	—	—
" 25	65.6	30.0	2,800	10.0	3,000	1,007	0.1	2,330	0.98	1.76	4.1	1.3	5.4	4.8	-0.6	—	—	—	—
" 26	65.4	30.0	2,798	10.0	3,000	1,005	Tr.	1,902	1.16	1.61	3.1	1.7	4.8	4.8	0	0.220	33.5	—	—
" 27	65.6	30.0	2,798	10.0	3,000	—	—	2,244	1.21	1.68	3.7	1.3	5.0	4.8	-0.2	—	—	210	130
" 28	65.4	30.0	2,793	10.0	3,000	—	Tr.	2,348	1.33	1.80	4.2	1.6	5.8	4.8	-1.0	—	—	—	—
" 29	65.0	30.0	2,793	10.0	3,000	1,007	0.3	1,873	1.37	2.00	3.7	1.5	5.2	4.8	-0.4	—	—	—	—
" 30	65.4	30.0	2,793	10.0	3,000	1,010	0.2	1,458	1.56	2.39	3.5	1.4	4.9	4.8	-0.1	0.211	21.4	—	—
Dec. 1	66.0	30.0	2,411	10.0	3,000	1,007	Tr.	1,896	1.72	2.49	4.7	1.3	6.0	9.6	+3.6	—	—	—	—
" 2	66.0	60.0	2,411	10.0	3,000	1,007	11.Tr.	2,249	1.62	2.29	5.2	2.1	7.3	9.6	+2.3	—	—	—	—
" 3	65.8	60.0	2,411	10.0	3,000	1,007	0.2	2,419	1.58	2.18	5.3	1.3	6.6	9.6	+3.0	0.272	19.1	—	—
" 4	65.8	60.0	2,411	10.0	3,000	1,007	—	2,319	1.84	2.40	5.6	2.3	7.9	9.6	+1.7	—	—	—	—
" 5	65.4	60.0	2,105	10.0	3,000	1,008	—	1,716	2.21	2.98	5.1	1.4	6.5	9.6	+3.1	—	—	—	—
" 6	65.8	60.0	1,995	10.0	3,000	1,009	0.2	1,598	2.56	3.32	5.3	1.4	6.7	9.6	+2.9	—	—	—	—
" 7	66.2	60.0	1,995	10.0	3,000	1,005	0.2	2,860	1.63	2.24	6.4	1.8	8.2	9.6	+1.4	0.363	19.2	200	120
" 8	65.4	60.0	1,995	10.0	3,000	1,010	0.4	1,637	2.72	3.59	5.9	1.1	7.0	9.6	+2.6	—	—	—	—
" 9	65.2	60.0	1,995	10.0	3,000	1,005	—	2,024	2.26	2.73	5.5	1.7	7.2	9.6	+2.4	—	—	180	120
" 10	65.8	60.0	1,995	10.0	3,000	1,006	—	2,412	1.93	2.52	6.0	2.0	8.0	9.6	+1.6	0.411	16.5	—	—
" 11	65.6	60.0	1,995	10.0	3,000	—	0.1	1,765	2.89	3.52	6.2	1.9	8.1	9.6	+1.5	—	—	—	—
" 12	65.6	60.0	1,995	10.0	3,000	—	0.1	2,710	2.15	2.42	6.5	1.8	8.3	9.6	+1.3	—	—	—	—
" 13	66.0	60.0	1,995	10.0	3,000	1,007	Tr.	2,051	2.37	3.08	6.3	1.2	7.5	9.6	+2.1	—	—	—	—
" 14	66.0	60.0	1,995	10.0	3,000	—	11.Tr.	2,292	2.32	2.81	6.45	1.9	8.35	9.6	+1.25	0.430	16.5	200	120

## UREA RETENTION IN NEPHRITIS

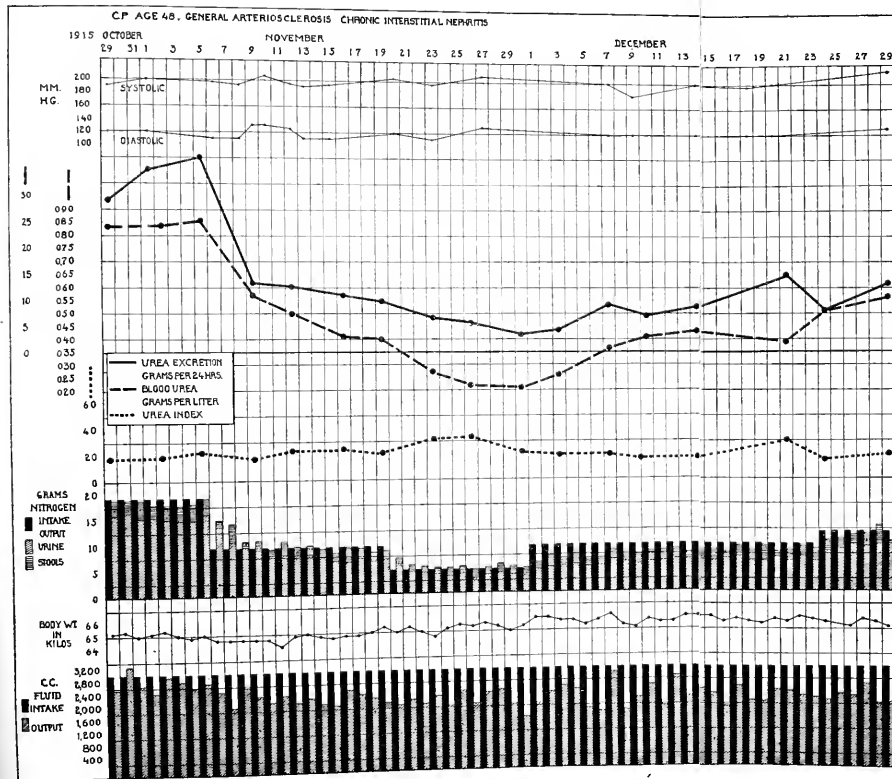
TABLE I—Concluded.

Date.	Weight.	Diet per 24 hrs.			Urine per 24 hrs.			Daily nitrogen balance.							Urea.		Blood pressure.		
		Protein.	Calories.	Sodium chloride.	Fluid.	Sp. gr.	Albumin per liter (Esbach).	Quantity.	Urea N per liter of urine.	Total N per liter of urine.	Total urine N in 24 hrs.	Stool N in 24 hrs.	Total N.			Urea per liter of blood.	Index of excretion (l).	Systolic.	Diastolic.
													Output in 24 hrs.	Intake in 24 hrs.	Balance.				
1915	kg.	gm.	gm.	gm.	cc.	gm.	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
Dec. 15	66.0	60.0	1,995	10.0	3,000	1,008	2,145	2.31	2.86	6.2	2.2	8.4	9.6	+1.2	—	—	—	—	
" 16	65.6	60.0	1,995	10.0	3,000	1,010	1,737	2.97	3.87	6.7	1.2	7.9	9.6	+1.7	—	—	—	—	
" 17	65.8	59.9	1,998	10.0	3,000	1,007	2,381	2.16	2.89	6.9	2.0	8.9	9.6	+0.7	—	—	—	—	
" 18	65.6	59.9	1,999	10.0	3,000	1,009	1,916	2.60	3.48	6.2	1.8	8.0	9.6	+1.6	—	—	—	195	
" 19	65.4	59.9	1,989	10.0	3,000	1,007	1,886	2.49	3.30	6.2	2.8	9.0	9.6	+0.6	—	—	—	120	
" 20	65.8	60.0	1,995	10.0	3,000	1,008	2,259	2.39	3.02	6.8	1.1	7.9	9.6	+1.7	—	—	—	200	
" 21	65.6	60.0	1,995	10.0	3,000	1,007	2,219	2.18	2.78	6.2	2.0	8.2	9.6	+1.4	0.388	30.0	—	120	
" 22	66.0	90.0	2,200	10.0	3,000	—	2,083	2.68	3.42	7.1	1.8	8.9	9.6	+0.7	—	—	—	—	
" 23	65.8	90.0	2,200	10.0	3,000	1,006	2,016	2.44	3.06	6.2	2.4	8.6	9.6	+1.0	—	—	—	—	
" 24	65.6	90.0	2,200	10.0	3,000	1,010	1,945	3.29	4.15	8.1	2.1	10.2	12.0	+1.8	0.506	15.0	—	—	
" 25	—	90.0	2,190	10.0	3,000	1,009	2,146	3.26	3.99	8.6	1.7	10.3	12.0	+1.7	—	—	—	—	
" 26	65.2	90.0	2,200	10.0	3,000	1,007	2,080	3.39	4.51	9.4	1.8	11.2	12.0	+0.8	—	—	—	—	
" 27	65.8	90.0	2,200	10.0	3,000	1,010	2,469	3.13	3.66	9.0	2.6	11.6	12.0	+0.4	—	—	—	—	
" 28	65.6	90.0	2,200	10.0	3,000	—	1,868	4.43	5.08	9.5	3.7	13.2	12.0	-1.2	—	—	—	—	
" 29	65.2	90.0	2,200	10.0	3,000	—	1,897	4.27	5.10	9.6	0.6	10.2	12.0	+1.8	0.564	19.8	220	130	





TEXT



TEXT-FIG. 1. Case 1. Graphic presentation of the data in Table I

of observation are given in Table I. The patient tolerated the strict dietary regimen well, and complained only of gastric distress at times.

At the time of discharge from the hospital, the patient had gained in weight and strength, but there had been no change in the urinary or blood findings.

*Case 2.*—W. F., male; age 17 years (Table II, Text-fig. 2).

*Diagnosis.*—Chronic interstitial nephritis, aortic and mitral stenosis.

Admitted to the hospital on March 27, 1916. The patient seemed quite well, but was brought to the hospital on account of albuminuria. This had been discovered 1 year before, after a brother aged 18 years had died suddenly of uremia. The patient had had three attacks of rheumatism, 6, 4, and 2 years ago. Following the last attack, he had heart failure, with edema of both legs, which had been relieved by administration of a drug which caused a marked diuresis. No history of scarlet fever, measles, or diphtheria was obtained.

On physical examination the patient appeared to be a normal healthy boy aged 17 years, well nourished, and with good color. The heart was enlarged, and there was a systolic murmur at the apex and a diastolic murmur at the base. There were no signs or symptoms of heart failure. The blood pressure was 140 systolic and 85 diastolic. The urine was clear, straw-colored, and acid; specific gravity 1,012. There was a heavy trace of albumin, and no sugar. The sediment contained hyaline casts, leukocytes, and occasional red blood cells.

The patient was kept under close observation, and the nitrogen balance determined from March 28 to June 11. During this time there was no change in the general condition. On May 31, 40 gm. of urea were added to the diet, on June 1, 40 gm., and on June 2, 20 gm. No effect was noticed except that the patient asked for an increase in the daily allowance of fluid. The patient stated that he felt no change in his condition on or after these days.

Since discharge from the hospital the general condition has remained unchanged. When last examined on November 22, 1916, the urine was amber and turbid; specific gravity 1,012. 1.2 gm. of albumin per liter of urine were found (Esbach). There were a few casts in the sediment. The blood urea concentration was 0.774 gm. per liter and the urea index was 28.2.

Since Cases 1 and 2 are exactly similar, they will be discussed together. Both show the essential features described by Widal and Javal in their case; that is, a close parallelism between nitrogen intake, concentration of urea in the blood, and nitrogen output. Changes in nitrogen intake were always quickly followed by a change in the concentration of urea in the blood and in nitrogen output, so that nitrogen balance was reestablished on the new level. In Case 2 extremely wide fluctuations in the concentration of urea in the blood were obtained, from a minimum of 0.262 gm. per liter to a maximum of 2.542 gm. It is significant that the upper limit was far above the

TABLE II.  
Case 2.

Date.	Weight kg.	Diet per 24 hrs.				Urine per 24 hrs.			Daily nitrogen balance.								Urea.		Blood pressure.				
		Protein. gm.	Calories.	Sodium chloride. gm.	Fluid. cc.	Sp. gr.	Albumin per liter (Esbach).	Quantity. cc.	Urea N per liter gm.	Total N per liter of urine. gm.	Total urine N in 24 hrs. gm.	Stool N in 24 hrs.			Total N.			Urea per liter of blood. gm.	Index of excre- tion (I).	Systolic.	Diastolic.		
												gm.	gm.	gm.	gm.	gm.	gm.					gm.	gm.
1916																							
Mar. 28	46.2	60.0	2,502	5.0	2,000	1.015	1.3	963	6.68	8.04	7.75	0.89	8.64	9.32	+0.68	0.610	22.5	140	85				
" 29	46.6	60.0	2,502	5.0	2,000	1.015	0.8	970	7.37	8.38	8.12	0.60	8.72	9.32	+0.60	—	—	140	90				
" 30	46.4	60.0	2,502	5.0	2,000	1.016	1.0	925	6.65	7.97	7.38	1.39	8.77	9.32	+0.55	—	—	130	85				
" 31	46.2	60.0	2,502	5.0	2,000	1.017	1.0	782	6.44	7.70	6.02	1.47	7.49	9.32	+1.83	0.524	35.0	150	90				
Apr. 1	46.4	60.0	2,502	5.0	2,500	1.015	0.5	1,095	4.75	5.70	6.25	0.87	7.12	9.32	+2.20	—	—	140	90				
" 2	47.2	60.0	2,502	5.0	2,500	1.011	0.2	1,415	4.34	4.74	6.71	0.13	6.84	9.32	+2.48	—	—	—	—				
" 3	47.6	60.0	2,502	5.0	2,500	1.007	0.4	2,196	2.56	3.09	6.78	2.42	9.20	9.32	+0.12	—	—	150	95				
" 4	47.0	60.0	2,502	5.0	2,500	1.009	0.3	1,589	3.21	3.78	6.01	1.14	7.15	9.32	+2.17	0.434	31.0	140	85				
" 5	46.0	60.0	2,502	5.0	2,500	1.009	0.3	1,751	2.96	3.62	6.34	2.32	8.66	9.32	+0.66	—	—	135	80				
" 6	47.0	60.0	2,502	5.0	2,500	1.009	0.5	1,731	2.69	3.33	5.76	—	5.76	9.32	+3.56	—	—	140	85				
" 7	46.8	60.0	2,502	5.0	2,500	1.010	0.2	1,557	3.45	4.05	6.30	0.86	7.16	9.32	+2.16	0.474	23.0	135	90				
" 8	47.2	60.0	2,502	5.0	2,500	1.009	0.3	1,750	3.13	3.65	6.39	2.36	8.75	9.32	+0.57	—	—	125	70				
" 9	47.0	60.0	2,502	5.0	2,500	1.009	0.3	1,656	3.36	3.89	6.45	0.62	7.07	9.32	+2.25	—	—	125	70				
" 10	46.8	60.0	2,500	5.0	2,500	1.011	0.4	1,831	3.92	4.60	8.41	5.04	13.45	9.32	-4.13	0.587	23.0	140	90				
" 11	47.0	60.0	2,500	5.0	2,500	1.010	0.2	1,505	3.74	4.40	6.66	0.98	7.64	9.32	+1.68	—	—	135	80				
" 12	47.2	60.0	2,500	5.0	2,500	1.010	0.3	1,565	3.65	4.24	6.65	1.49	8.14	9.32	+1.18	—	—	135	80				
" 13	47.0	60.0	2,500	5.0	2,500	1.012	0.25	1,300	3.98	5.02	6.53	1.50	8.03	9.32	+1.29	—	—	140	80				
" 14	47.0	60.0	2,500	5.0	2,500	1.011	0.5	1,141	4.11	5.06	5.78	0.78	6.56	9.32	+2.76	0.559	26.8	135	90				
" 15	47.4	60.0	2,500	5.0	2,500	1.013	0.35	1,050	4.48	5.64	5.93	2.24	8.17	9.32	+1.15	—	—	130	80				
" 16	47.0	60.0	2,500	5.0	2,500	1.011	0.2	1,068	4.52	5.65	6.03	1.76	7.79	9.32	+1.53	—	—	135	80				
" 17	47.0	60.0	2,500	5.0	2,500	1.012	0.4	1,331	3.82	4.91	6.54	1.91	8.45	9.32	+0.87	—	—	135	80				

Apr. 18	47.4	60.0	2,500	5.0	2,500	1,012	0.25	1,274	3.79	4.91	6.12	2.42	8.54	9.32	+0.78	0.562	29.0	135
" 19	47.6	60.0	2,503	5.0	2,500	1,011	0.7	1,066	3.23	4.16	4.44	1.46	5.90	4.53	-1.37	—	—	135
" 20	47.6	60.0	2,503	5.0	2,500	1,009	0.2	1,435	2.28	2.97	4.25	1.76	6.01	4.53	-1.48	—	—	135
" 21	47.8	60.0	2,503	5.0	2,500	1,008	0.2	1,537	2.04	2.63	4.04	1.10	5.14	4.53	-0.61	0.377	28.0	135
" 22	48.0	60.0	2,503	5.0	2,500	1,008	0.3	1,720	1.68	2.20	3.78	2.09	5.87	4.53	-1.34	—	—	145
" 23	47.2	60.0	2,503	5.0	2,500	1,007	0.2	1,375	1.69	2.27	3.12	1.71	4.83	4.53	-0.30	—	—	130
" 24	48.0	30.0	2,503	5.0	2,500	1,010	0.2	1,243	1.62	2.37	2.95	1.55	4.50	4.53	+0.03	0.288	26.0	140
" 25	47.8	30.0	2,503	5.0	2,500	1,007	0.5	1,355	1.61	2.25	3.05	0.37	3.42	4.53	+1.11	—	—	135
" 26	48.0	30.0	2,503	5.0	2,500	1,007	0.3	1,684	1.32	1.87	3.15	1.68	4.83	4.53	-0.30	—	—	150
" 27	48.0	30.0	2,503	5.0	2,500	1,006	0.2	1,220	1.66	2.26	2.76	2.16	4.92	4.53	-0.39	0.287	29.5	140
" 28	48.0	15.0	2,501	5.0	2,500	1,007	0.3	1,941	1.06	1.47	2.85	0.98	3.83	2.40	-1.43	—	—	140
" 29	48.0	15.0	2,501	5.0	2,500	1,008	0.35	1,225	1.52	2.16	2.64	0.69	3.33	2.40	-0.93	—	—	150
" 30	47.8	15.0	2,501	5.0	2,500	1,006	0.3	1,470	1.20	1.66	2.44	1.15	3.59	2.40	-1.19	—	—	145
May 1	47.8	15.0	2,501	5.0	2,500	1,007	0.25	2,056	1.04	1.42	2.92	0.63	3.55	2.40	-1.15	0.262	26.0	145
" 2	47.8	75.0	2,499	5.0	2,500	1,011	0.4	1,216	1.90	2.75	3.34	1.38	4.72	11.5	+6.78	—	—	145
" 3	47.6	75.0	2,499	5.0	2,500	1,011	0.5	1,146	2.68	3.43	3.93	2.48	6.41	11.5	+5.09	—	—	140
" 4	48.0	75.0	2,499	5.0	2,500	1,009	0.3	1,480	2.72	3.38	5.00	2.38	7.38	11.5	+4.12	0.485	21.0	150
" 5	48.0	75.0	2,499	5.0	2,500	1,008	0.2	1,717	2.77	3.31	5.68	2.19	7.87	11.5	+3.63	—	—	140
" 6	48.2	75.0	2,499	5.0	2,500	1,009	0.2	1,858	2.60	3.17	5.90	0.90	6.80	11.5	+4.70	—	—	135
" 7	48.0	75.0	2,499	5.0	2,500	1,009	0.3	1,540	3.23	4.00	6.15	1.10	7.25	11.5	+4.25	—	—	140
" 8	48.4	75.0	2,499	5.0	2,500	1,010	0.25	1,751	3.11	3.82	6.69	2.06	8.75	11.5	+2.75	0.656	13.3	132
" 9	48.8	75.0	2,499	5.0	2,500	1,010	0.4	2,286	2.34	2.99	6.85	0.71	7.56	11.5	+3.94	—	—	145
" 10	48.6	75.0	2,499	5.0	2,500	1,008	0.4	1,557	3.44	4.16	6.46	2.17	8.63	11.5	+2.87	—	—	140
" 11	48.8	75.0	2,499	5.0	2,500	1,007	0.5	1,686	3.66	4.47	7.54	2.04	9.55	11.5	+1.95	—	—	140
" 12	48.6	75.0	2,499	5.0	2,500	1,010	0.5	1,483	4.18	4.97	7.39	4.01	11.43	11.5	+0.07	0.603	25.5	—
" 13	48.6	75.0	2,499	5.0	2,500	1,010	0.45	1,634	4.29	4.98	8.15	1.91	10.06	11.5	+1.44	—	—	140
" 14	48.6	75.0	2,499	5.0	2,500	1,009	0.5	1,377	4.44	5.34	7.35	0.62	7.97	11.5	+3.53	—	—	—
" 15	48.6	75.0	2,499	5.0	2,500	1,008	0.5	1,378	4.26	5.16	7.11	1.48	8.59	11.5	+2.91	—	—	145
" 16	48.6	75.0	2,499	5.0	2,500	1,007	0.45	1,988	3.26	3.84	7.63	2.24	9.87	11.5	+1.63	0.611	22.5	135
" 17	48.6	120.0	1,782	5.0	2,500	1,008	0.45	1,498	4.08	4.81	7.20	2.56	9.76	18.4	+8.64	—	—	135
" 18	49.0	120.0	1,782	5.0	2,500	1,009	0.6	2,024	4.66	5.50	11.13	3.00	14.13	18.4	+4.27	—	—	145

TABLE II—*Concluded.*

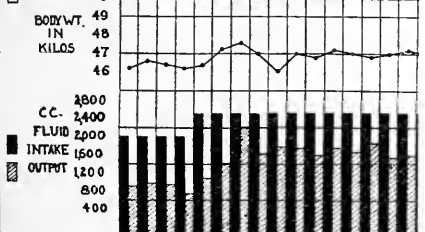
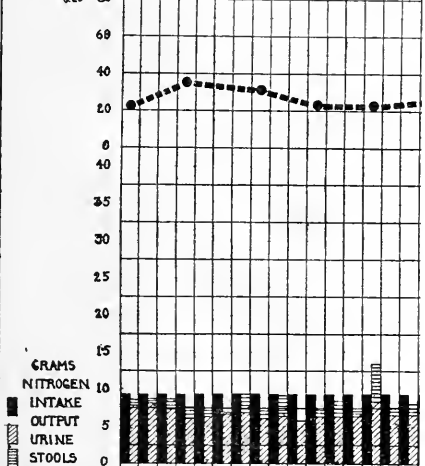
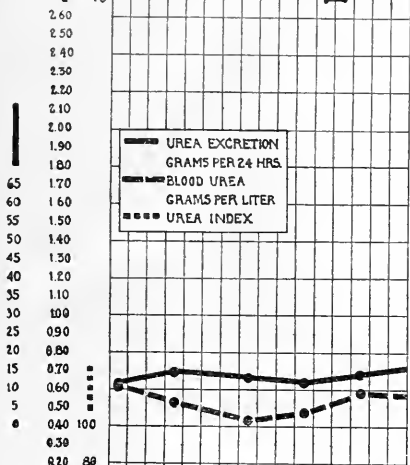
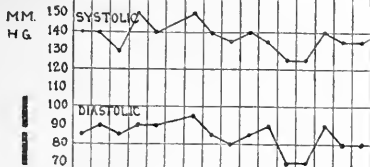
Date.	Weight.	Diet per 24 hrs.				Urine per 24 hrs.				Daily nitrogen balance.								Urea.		Blood pressure.	
		Protein.		Calories.	Sodium chloride.	Fluid.	Sp. gr.	Albumin per liter (Esbach).	Quantity.	Urea N per liter	Total N per liter of urine.	Total urine N in 24 hrs.	Stool N in 24 hrs.	Total N.			Urea per liter of blood.	Index of excretion (l).	Systolic.	Diastolic.	
		gm.	kg.	gm.	cc.	gm.	gm.	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
1916																					
May 19	49.2	120.0	1,782	5.0	2,500	1,010	0.5	1,921	5.24	6.08	11.70	2.56	14.26	18.4	+4.14	0.808	14.3	—	—		
" 20	49.0	120.0	1,502	5.0	2,500	1,012	0.5	1,802	6.38	7.23	13.02	1.14	14.16	18.4	+4.24	—	—	130	80		
" 21	49.0	120.0	1,502	5.0	2,500	1,010	0.5	1,826	6.65	7.39	13.50	1.82	15.32	18.4	+3.08	—	—	120	80		
" 22	48.8	120.0	1,502	5.0	2,500	1,010	0.3	1,937	6.45	7.22	14.00	1.60	15.6	18.4	+2.8	—	—	140	90		
" 23	48.2	120.0	1,502	5.0	2,500	1,011	0.3	1,775	6.92	7.62	13.52	3.66	17.18	18.4	+1.22	1.263	13.5	125	80		
" 24	48.4	120.0	1,502	5.0	2,500	1,010	0.3	1,903	7.26	8.03	15.3	—	15.3	18.4	+3.10	—	—	140	90		
" 25	49.0	120.0	1,502	5.0	2,500	1,011	0.5	1,778	7.75	8.44	15.0	1.68	16.68	18.4	+1.72	—	—	120	70		
" 26	48.2	120.0	1,502	5.0	2,500	1,011	0.2	1,992	7.65	8.52	17.0	2.52	19.52	18.4	—1.12	1.314	18.1	140	90		
" 27	48.0	120.0	1,502	5.0	2,500	1,014	0.2	1,930	8.00	8.66	16.70	1.95	18.65	18.4	—0.25	—	—	128	72		
" 28	48.0	120.0	1,502	5.0	2,500	1,012	0.4	1,582	8.21	9.05	14.32	—	14.32	18.4	+4.08	—	—	—	—		
" 29	47.6	120.0	1,502	5.0	2,500	1,010	0.3	2,420	6.46	7.04	17.02	0.73	17.75	18.4	+0.65	—	—	125	80		
" 30	48.0	120.0	1,502	5.0	2,500	1,011	0.45	1,753	7.75	8.49	14.90	2.38	17.28	18.4	+1.12	1.192	18.8	125	80		
" 31	48.0	120.0*	1,502	5.0	2,500	1,011	0.2	2,055	8.26	8.89	18.25	1.45	19.70	36.8	+7.1	—	—	120	75		
June 1	47.8	120.0*	1,502	5.0	2,500	1,009	0.2	2,515	9.68	10.70	26.9	3.36	30.26	36.8	+6.54	—	—	125	80		
" 2	47.0	120.0†	1,502	5.0	2,500	1,011	0.3	2,558	10.21	11.31	28.9	1.87	30.77	27.6	—3.17	2.542	8.3	120	75		
" 3	46.6	60.0	2,502	5.0	2,800	1,010	0.3	1,548	9.65	10.40	16.1	1.32	17.42	9.32	—8.10	—	—	125	80		
" 4	47.4	60.0	2,502	5.0	2,800	1,010	0.4	1,586	7.32	8.12	12.85	1.36	14.2	9.32	—4.88	—	—	130	75		
" 5	48.0	60.0	2,502	5.0	2,800	1,008	0.5	1,812	4.27	5.18	9.40	2.04	11.44	9.32	—2.12	0.903	14.5	130	75		
" 6	48.0	60.0	2,502	5.0	2,800	1,007	0.3	2,066	3.27	5.37	10.92	1.46	12.38	9.32	—3.06	—	—	130	75		
" 7	48.2	60.0	2,502	5.0	2,800	1,006	0.3	1,647	2.66	4.75	7.82	4.3	12.12	9.32	—2.80	—	—	130	85		
" 8	48.4	60.0	2,502	5.0	2,800	1,006	0.3	2,296	2.15	4.05	9.30	2.98	12.28	9.32	—2.96	0.470	22.2	135	80		
" 9	48.6	60.0	2,502	5.0	2,800	1,007	0.2	2,142	2.34	4.07	8.72	1.44	10.16	9.32	—0.84	—	—	130	80		
" 10	48.6	60.0	2,502	5.0	2,800	1,006	0.3	2,218	2.22	4.07	9.02	2.49	11.51	9.32	—2.19	—	—	130	80		
" 11	48.6	60.0	2,502	5.0	2,800	1,006	0.2	2,135	2.18	4.08	8.72	1.79	10.51	9.32	—1.19	0.455	19.6	135	75		

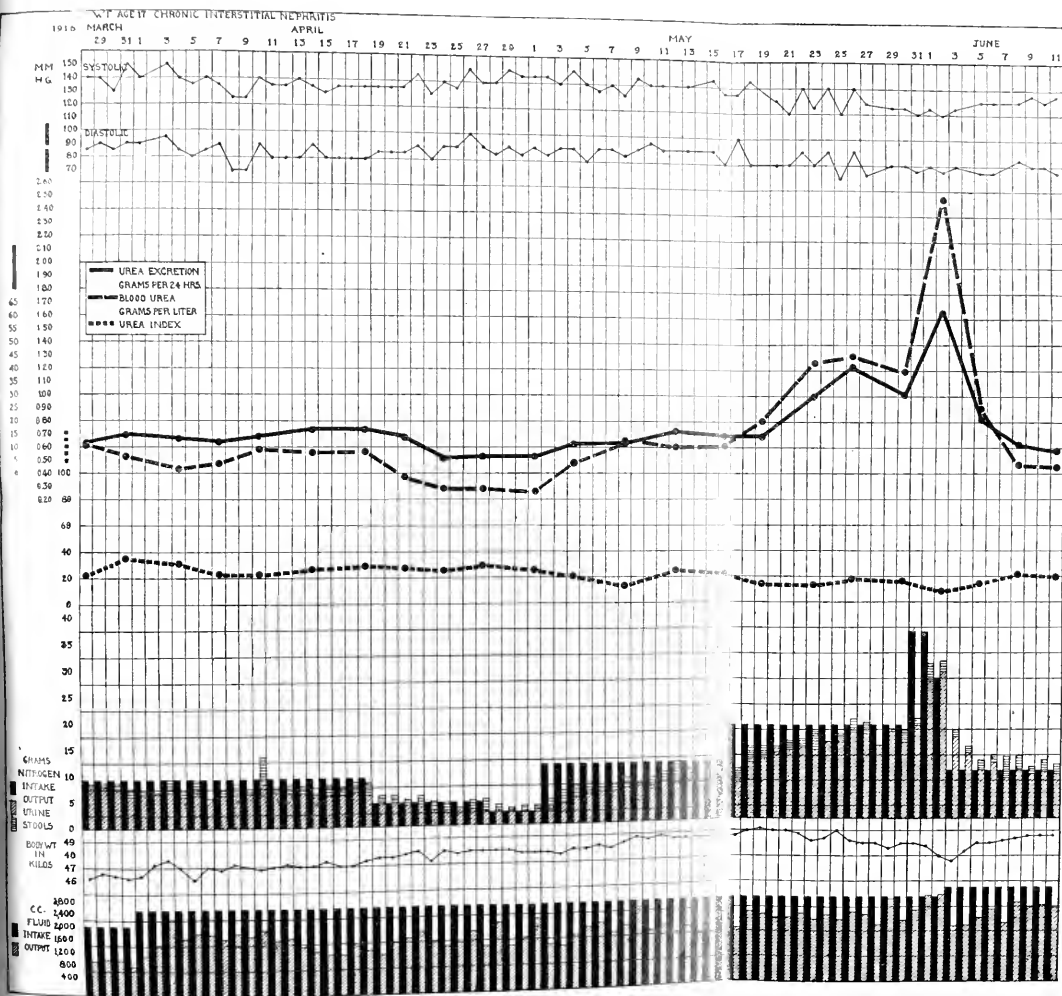
\*40 gm. of urea added to diet.

†20 gm. of urea added to diet.

W.F. AGE 17 CHRONIC INTERSTITIAL N  
1916 MARCH

29 31 1 3 5 7 9 11





TEXT-FIG. 2. Case 2. Graphic presentation of the data in Table II.



concentration in the blood at which Hewlett, Gilbert, and Wickett (4) believe that toxic effects of urea appear. The subject in this case is one in whom uremic symptoms should appear when the blood urea rises to a high level, if these symptoms are in any way referable to urea. As a matter of fact, no effect was noted beyond an increased fluid output, a slight loss in weight, and increased thirst.

The findings regarding urea excretion are of especial interest. In Case 1, with a variation in blood urea concentration from a minimum of 0.211 gm. per liter to a maximum of 0.858 gm., the index of urea excretion remained remarkably constant. In eighteen observations, with an average of 21.4, a figure below 16.4 is noted only once, and above 26.4 three times. The variations must be regarded as insignificant when the complexity of the formula and the possibility of error in obtaining the final result are taken into account.

In Case 2 a somewhat greater range of variation is noted. The most significant variation is that seen on June 2, when the blood urea reached 2.542 gm. per liter. Apparently the demand on the kidneys for the excretion of urea had exceeded their maximum capacity, and an actual accumulation had begun. This occurred, however, only when the nitrogen intake had been maintained at over 36 gm. per day for more than 2 days.

The two cases confirm the findings of Widal and Javal, and add the fact that no essential variation in the ability of the kidneys to respond to even high concentrations of urea in the blood at various levels of protein metabolism was demonstrable, until a very high level was reached; that is, the quantitative relationship that existed between the concentration of urea in the blood and the rate of its excretion remained the same at all levels of protein metabolism. The usual increase in blood urea concentration in nephritic individuals, on a liberal allowance of protein, can, therefore, hardly be attributed to diminished capacity for urea elimination. In these patients the capacity for urea excretion was far beyond the ordinary demands on that function, yet a relative increase in the blood urea concentration is evident at all levels of protein metabolism.

Several writers who have published observations obtained by means of Ambard's laws have emphasized the variations which occur in normal individuals. These variations, which may be quite wide,

are known to occur in the same individual and among different individuals. Attention has repeatedly been called to similar observations in previous papers (3). These variations, however, do not appear to affect the validity of Ambard's laws. Evidence that the laws are valid in as far as the variables considered are concerned seems conclusive. If this is true, then variations in the results must be accounted for by changes in the individual which introduce certain variable factors not represented in the formula. We have previously suggested that the variation may be due in part to such variable factors.

In the first place, it has been generally recognized that the results are more nearly constant in nephritic individuals than in normal individuals. The cases outlined above illustrate the constancy of the laws in two individuals with a marked disturbance in the elimination of urea. It is in normal individuals, as has already been noted, that a marked variability occurs in the index, although the findings do not, as a rule, give rise to doubt as to whether the individual falls within a normal or an abnormal group. Variability or elasticity of this sort probably represents a factor of safety. Its loss, therefore, would seem to be a sign of disease, even though this loss occurs before any quantitative diminution in renal function appears. If this assumption is correct, we should expect to find individuals in whom normal, or nearly normal quantitative estimations of renal function are found, but in whom, on repeated examination, there is the striking constancy in numerical results of the application of Ambard's laws that is shown by distinctly abnormal cases. These individuals would not, then, be considered normal. Cases 3 to 6 (Tables III to VI) show this constancy in numerical value of the index.

*Case 3.*—E. C., female; age 39 years (Table III).

*Diagnosis.*—Mitral stenosis, arterial hypertension, hypertrophy of heart.

The patient was under observation from December 2, 1915. She complained of headache, cough, and dyspnea on exertion, and presented the physical signs of mitral stenosis. There was no edema. Her blood pressure varied while under observation from 210 systolic and 130 diastolic, to 140 systolic and 80 diastolic after rest in bed. Examination of the urine on December 11 showed it to be clear, amber, acid, and to contain a very faint trace of albumin but no casts or

sugar; specific gravity 1.027. On repeated examination the amount of albumin varied from negative to a trace. Hyaline casts were found but once. The patient was subjected to alterations in diet, but the nitrogen balance was not determined. Her general condition improved with complete rest, but there was no change in the physical signs, except for lowering of blood pressure.

The total elimination of phenolsulfonephthalein on December 2 was 65 per cent in 2 hours.

The findings regarding urea excretion in the six observations which were made are tabulated in order, according to the concentration of urea found in the blood.

TABLE III.

## Case 3.

Date.	Weight.	Urine in 24 hrs.	Urea.			Index of excretion (1°).
			Per liter of blood (U.*).	Per liter of urine (C*).	Amount in 24 hrs. (D*).	
<i>1915-16</i>	<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Dec. 31.....	72.2	7,100	0.107	1.30	9.07	112
Jan. 26.....	69.6	9,300	0.170	1.69	15.7	91
Dec. 3.....	76.0	4,900	0.187	3.13	15.3	92
" 2.....	75.5	10,200	0.196	2.23	22.8	105
Jan. 11.....	72.0	2,020	0.252	8.82	17.85	104
Dec. 14.....	74.6	2,600	0.300	9.35	24.3	99

\* For the explanation of these abbreviations see the formula for the index of urea excretion.<sup>1</sup>

Case 4.—J. F., male; age 43 years (Table IV).

*Diagnosis.*—Mitral stenosis, auricular fibrillation, chronic heart failure.

The patient was admitted on November 27, 1915, and remained in the hospital until July 11, 1916, when he died. During the entire stay he presented a typical picture of chronic cardiac valvular disease with heart failure. Response to treatment was at times striking, but never prolonged, and the patient was never entirely free from edema. The amount of albumin in the urine varied from a very faint trace, at times when the elimination of urine was best, to 3.0 gm. per liter (Esbach) at times when the other signs of cardiac failure, such as dyspnea, cyanosis, edema, and scanty urine, were worst. Hyaline and granular casts were present in abundance at times, but were few in number or absent when the urine albumin was low in amount. Red blood cells of undetermined origin were constantly present. The findings regarding urea excretion also varied with the condition of the patient, quantitatively normal findings being present at times when other signs were favorable. The table shows four successive observations made during one of these periods. At this time the patient was voiding an amount

of fluid in excess of his intake, felt fairly well, and the edema was disappearing. The urine on March 11, was amber, turbid, and there was a very faint trace of albumin; specific gravity 1,019. The sediment contained red blood cells, but no casts were seen.

TABLE IV.

*Case 4.*

Date.	Weight.	Urine in 24 hrs.	Urea.			Index of excretion (I).
			Per liter of blood (Ur.).	Per liter of urine (C).	Amount in 24 hrs. (D).	
<i>1916</i>	<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Mar. 7.....	63.0	1,220	0.345	18.15	22.1	112
" 8.....	62.6	900	0.294	17.65	15.9	108
" 9.....	62.0	1,200	0.279	13.4	16.1	110
" 12.....	60.8	1,280	0.333	17.2	22.0	121

*Case 5.*—M. G., male; age 58 years (Table V).

*Diagnosis.*—General arteriosclerosis, chronic myocarditis, and hypertrophy of heart.

The patient was admitted to the hospital on December 8, 1915, and remained under observation until April 3, 1916. On admission he complained of swelling of the feet and dyspnea on exertion. There was marked edema of both lower extremities. The heart was considerably enlarged, the rate was rapid, and the rhythm normal. A systolic murmur was heard at the apex. The urine was turbid, dark amber, acid, and contained 0.25 gm. of albumin per liter (Esbach); specific gravity 1,013. There was no sugar. The sediment contained many leukocytes and leukocytic and hyaline casts. After a few days in bed without change in condition a marked diuresis was induced with theocin (0.9 gm. daily for 3 days). The edema of the extremities rapidly disappeared, there being practically none demonstrable on December 17, although fluid in the chest persisted for some time longer. At this time there was only a faint trace of albumin in the urine, with numerous leukocytes, but no casts in the sediment. The general condition of the patient remained good. The table shows all observations made on urea excretion from the time of admission until January 5, 1915, when theocin was again administered. The patient later had an attack of hemiplegia, and one of bronchopneumonia, but was discharged from the hospital in fairly good condition on March 5, 1916. He died on May 31, of a second attack of hemiplegia.

TABLE V.

Case 5.

Date.	Weight.	Urine in 24 hrs.	Urea.			Index of excretion (I).	Medication.
			Per liter of blood (Ur.).	Per liter of urine (C).	Amount in 24 hrs. (D).		
<i>1915</i>	<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		
Dec. 9	91.6	1,060	0.403	19.43	20.6	55	
" 12	91.6	2,000	0.374	13.8	27.6	72	
" 13	91.0	1,535	0.381	14.1	21.7	55	
" 14	89.2	2,840	0.336	7.78	22.1	55	Theocin.
" 15	86.4	5,360	0.232	6.4	34.2	167	"
" 16	83.8	3,400	0.234	6.98	23.7	123	"
" 20	82.2	2,160	0.384	14.1	30.4	85	
" 23	80.2	2,360	0.361	12.1	28.6	85	
" 27	78.0	1,840	0.461	19.6	36.0	86	
" 30	78.0	1,620	0.440	20.7	36.6	89	
<i>1916</i>							
Jan. 3	78.6	2,000	0.457	19.85	39.7	97	
" 5	77.8	1,800	0.416	17.4	31.3	87	

Case 6.—J. A., male; age 43 years (Table VI).

*Diagnosis.*—Chronic interstitial nephritis, hypertrophy of heart.

The patient was first admitted to the hospital in March, 1915, and has been under almost constant observation since that time. He suffers from frequent attacks of cardiac insufficiency associated with severe edema, ascites, and hydrothorax. He responds readily to treatment, and generally leaves the hospital in fairly good condition, without dyspnea, cyanosis, or edema. Table VI records the observations made during the patient's third stay in the hospital, and all the findings of the period from March 23 to April 20 are shown. An unusual constancy in the index of excretion is shown. During this time the patient was improving rapidly while taking digipuratum, 0.2 gm., and diuretin, 0.2 gm. daily, 5 days out of 7. The urine was clear, amber, and acid; specific gravity 1,009. There was a trace of albumin, and a few hyaline casts were seen in the sediment. Fluid excretion during the period was constantly in excess of the intake. At other times the findings have shown a marked difference from those detailed below. The patient is at present again in the hospital, having been admitted for the fifth time.

TABLE VI.

*Case 6.*

Date.	Weight.	Urine in 24 hrs.	Urea.			Index of excretion (I).
			Per liter of blood (Ur.).	Per liter of urine (C).	Amount in 24 hrs. (D).	
<i>1916</i>	<i>kg.</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Mar. 23.....	85.6	1,745	0.326	9.53	16.62	50
" 24.....	84.8	1,300	0.312	11.6	15.1	56
" 25.....	84.0	2,600	0.310	7.46	19.4	59
" 27.....	78.6	1,400	0.310	9.4	13.15	48
" 28.....	77.2	940	0.300	12.22	11.5	52
" 30.....	76.6	860	0.337	14.52	12.5	51
Apr. 1.....	76.0	1,100	0.340	11.95	13.15	46
" 3.....	76.6	1,200	0.407	16.4	19.65	56
" 4.....	76.8	1,200	0.423	17.25	20.7	56
" 6.....	74.8	1,100	0.399	15.55	17.1	51
" 8.....	72.0	700	0.354	18.15	12.7	54
" 10.....	71.8	1,015	0.447	18.8	19.1	52
" 13.....	72.2	1,300	0.507	19.2	24.95	52
" 16.....	72.0	940	0.411	16.35	15.4	46
" 18.....	71.0	900	0.414	17.8	16.05	50
" 20.....	69.8	800	0.329	15.8	12.6	59

Cases 3, 4, and 5 show results regarding urea excretion resembling the normal as far as numerical value is concerned, but differing from healthy individuals in that they show no tendency to variation in the numerical value of the index. The significance of such findings has not yet been determined, but they have a distinct bearing on the physiology of urea excretion. We assume that as a result of some unknown pathological change, a fixation of certain factors involved in urea excretion which are variable in normal individuals has occurred. The numerical results remain constant, even when wide variations in the level of protein metabolism are brought about by changes in the diet. This was shown to be true in Case 3. Case 6 shows findings more definitely pathological, but here too is a high degree of constancy in the index.

In Case 5, during a time when the circulation was obviously poor and the urine showed evidence of renal passive congestion, the index was nearly constant at 55. The administration of theocin was followed by marked diuresis and a temporary increase in the index.

The edema disappeared as well as the signs of passive congestion. The index then remained constant at about 85. The index was accordingly capable of variation, but not in the manner that variation ordinarily occurs in normal individuals. One must conclude that the factors which are usually variable in normal individuals were unusually fixed in this individual. Case 4 resembled Case 5.

Case 3 was one of those we are accustomed to include in the group of primary hypertension, in view of the absence of demonstrable renal lesion. According to the view just presented, the kidneys of this patient have lost their normal elasticity. Whether this loss represents the forerunner of an actual impairment of renal function must be left for further investigation to determine.

#### DISCUSSION.

The experimental facts presented demonstrate that the occurrence of a relatively increased concentration of urea in the blood follows the increased resistance with which diseased kidneys oppose the passage of urea. When, as the result of feeding an increased amount of nitrogen, the concentration of urea in the blood rises, a parallel increase in the rate of excretion of urea occurs. When a point is reached at which the rate of urea excretion is kept equal to the rate of formation by the organism, the level of urea in the blood ceases to rise and the organism remains in nitrogen equilibrium. But when the nitrogen intake in this individual is diminished, urea is excreted for a time more rapidly than it is formed, until the level of urea in the blood falls and reaches a point such that the rate of urea excretion is again equal to the rate of urea formation, and the organism is once more in nitrogen equilibrium. The experiments illustrate the events occurring in the so called retention of urea.

Fluctuation in the level of urea in the blood thus occurs in abnormal individuals in exactly the same way in which it occurs in normal individuals. The mechanism of excretion remains the same, but on account of the increased resistance with which the kidneys oppose the passage of urea the rate of urea excretion becomes relatively less rapid than in the case of normal individuals, and the level of urea in the blood becomes relatively higher. It is the rate of urea excretion, relative to the other conditions found and relative to the usual nor-

mal<sup>1</sup>rate, that the index of urea excretion, based on Ambard's laws, expresses in numerical form.

As has been shown, the index of urea excretion may vary within wide limits in normal persons. These limits may be known as the limits of normality, and their extremes usually lie between 80 and 200. Fluctuations to this extent must, we think, be emphasized as well as the degree of constancy on which insistence has until now been placed, and which originally supplied the evidence on which the laws of Ambard were based.

In certain individuals, however, not fluctuation but extreme constancy, to which we now apply the term fixation, is the rule. Fixation is, in some cases, too striking to be considered as due to coincidence. It is dwelt upon in this paper on account of the support which it gives to the laws of Ambard. It appears now to be desirable to distinguish between extreme constancy or fixation and the relative constancy which we find in normal persons. The relative constancy of normal individuals is intended to include, as has been stated, a certain fluctuation as far as numerical values are concerned. It must be taken to include that degree of fluctuation, almost always above the level of 80, which normal people often exhibit. Fluctuation of this type has been found by all observers and is greater than the range of experimental error. The cause and the significance of fluctuation is difficult to determine. It may depend either upon the influence of variable factors not determined as yet, and therefore not included in the formula, or on the fact that the ascertainable values now included in the formula do not hold that relation to each other which the laws imply. The latter interpretation we hold to be inadmissible because there is a sufficient degree of constancy, even under conditions where fluctuation occurs, to furnish strong evidence of the validity of the laws. In certain abnormal conditions where fixation has been shown to occur, the striking degree of constancy obtained must be interpreted as adding greatly to this evidence already furnished by the degree of constancy obtained in normal individuals. The pathological significance of this fixation, especially when it occurs within the limits of normality, is not yet determined.

Finally, we have failed to note uremic symptoms in a patient in whom the concentration of urea in the blood rose, under experimental conditions, to 2.542 gm. per liter.



## CONCLUSIONS.

1. Urea retention, in the sense of a relatively increased concentration in the blood, is the result of increased resistance to the excretion of urea through the kidneys.

2. The relatively increased concentration of urea in the blood overcomes the increased resistance to excretion, and the organism is thereby maintained in nitrogenous equilibrium.

3. The laws formulated by Ambard for the excretion of urea apply in the condition of urea retention under a widely varying range of conditions, as to nitrogen intake and excretion.

4. The numerical value of Ambard's constant changes in urea retention, but the relation of the variable factors to one another remains otherwise unchanged.

5. In certain individuals, with otherwise normal findings in regard to urea excretion, an unusual degree of constancy, to which we have applied the term fixation, has been found in the numerical results obtained by the application of Ambard's laws. These individuals are regarded, as the result of this study, as probably abnormal, but the pathological significance of the fixation has not been determined.

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# EXPERIMENTS ON THE CAUSATION AND AMELIORATION OF ADRENALIN PULMONARY EDEMA.

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PLATES 14 TO 16.

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During an investigation of the absorption of drugs<sup>1</sup> by the pulmonary air passages of the rabbit we observed that an intratracheal injection of adrenalin, after a double vagotomy, apparently produced a fulminant pulmonary edema much more readily than when the vagi were intact. In the present paper we shall report a study of this subject, together with other series of experiments, which finally led to the recognition of a practically ignored respiratory factor in the production of adrenalin pulmonary edema.

That adrenalin may cause pulmonary edema in the normal rabbit was noted early in the experimental investigation of this substance. Bouchard and Claude<sup>2</sup> in 1902 observed pulmonary edema and death in the normal rabbit after the intravenous injection of adrenalin, an observation abundantly corroborated. Pulmonary edema in the rabbit may also be obtained when the adrenalin is injected subcutaneously, though tremendous doses are then necessary; thus Battelli and Taramasio<sup>3</sup> produced this effect with the subcutaneous injection of 10 mg. of adrenalin per kilo.

The facilitating action of vagus section upon the occurrence of adrenalin pulmonary edema in the rabbit has not to our knowledge been observed before. In artificially induced hydremic plethora, however, the vagi, according to Kraus,<sup>4</sup> play in this respect a decisive part.

<sup>1</sup> Auer, J., and Gates, F. L., *J. Exp. Med.*, 1916, xxiii, 757.

<sup>2</sup> Bouchard, C., and Claude, H., *Compt. rend. Acad.*, 1902, cxxv, 928.

<sup>3</sup> Battelli, F., and Taramasio, P., *Compt. rend. Soc. biol.*, 1902, liv, 815.

<sup>4</sup> Kraus, F., *Z. exp. Path. u. Therap.*, 1913, xiv, 402.

## EXPERIMENTAL.

*Methods.*

Rabbits only were used in this investigation. The animals were placed on an electric warming pad and ether anesthesia was employed for operative procedures. A wide glass tube about 2 cm. long was tied into the trachea. When blood pressure was recorded the right carotid artery was connected to the mercury manometer by tubing filled with half saturated sodium sulfate. When the lungs and heart were inspected the sternum was split longitudinally; usually both pleural sacs were opened. Intratracheal insufflation was then started so that the lungs were always moderately distended. The vagi, when sectioned, were divided in the neck.

The adrenalin employed was the ordinary commercial product obtained in the market, and was slowly injected by syringe through the tracheal tube. The dose was usually 0.25 cc. per kilo of body weight; occasionally more or less was used.

When atropine was administered, this drug was usually injected intratracheally; in a few instances the atropine was injected intramuscularly.

As criteria for the presence of pulmonary edema we utilized the appearance of foam in the trachea and the autopsy findings. As a rule, the medulla was punctured in the survivors 30 minutes after the injection; in some instances 1 to 3 hours were allowed to elapse.

Although the appearance of the typical tracheal foam is positive proof of the existence of pulmonary edema, yet its absence by no means indicates the absence of pulmonary edema. Râles may be audible to the unaided ear and easily felt by the fingers and yet the trachea may remain perfectly clear of foam. This happens especially when the injected animals do not struggle; there is then no mechanical compression of the lungs and the foam and fluid remain in the lower air passages. For this reason we killed the surviving animals after a period of time by medullary puncture and at once examined the lungs.

Several series of experiments were carried out. In the first series both vagi were divided and after not less than 10 minutes the adrenalin was injected. The controls for this series were normal animals with intact vagi, but in many of them the adrenalin was injected repeatedly, thus rendering the control test especially severe.

In another series with divided vagi, the behavior of the heart was examined by inspection, the sternum being split lengthwise and the pericardial sac opened. Artificial respiration was instituted before the lungs were exposed. The adrenalin was always administered repeatedly. In a subsidiary series artificial respiration, with and

without ether, was given to rabbits with chests intact but with vagi divided.

In still another series the vagi were again divided, but atropine was administered before the injection of adrenalin.

In a final series the effect of tracheal stenosis upon the production of pulmonary edema in normal rabbits (vagi intact) was tested.

### *Results in Series with Divided Vagi.*

The vagi were divided before the adrenalin was injected. The average dose was 0.25 cc. per kilo, and as a rule only one injection was given. The following protocol will give a clear picture of a typical experiment.

*Rabbit 1.*—Gray male; weight 1,740 gm.

10.45. On electric pad at medium.

10.58. Start ether.

11.02. Operation completed; both vagi divided, cannula in trachea. Stop ether.

11.08. Respiration rapid, easy. No inspiratory stoppage. Heart moderately rapid, regular, good strength.

11.10. Change pad to low.

+11.12. 0.52 cc. adrenalin into trachea (0.3 cc. per kilo); no loss.

11.13. Respiration more shallow and more rapid.

11.13½. Respiration slowed, deeper, with retraction of costal margin; respiration stopped.

11.14½. Respiration starts, slow, deep, with retraction of costal margins. Heart very rapid, apparently regular, good strength.

11.15. Struggle; pink foam in tracheal tube.

11.15¼. Pink foam pouring from tracheal tube.

11.16. Struggle, marked outflow of pink foam. Heart apparently regular, rapid (palpation).

11.17. Respiration rapid; strong retraction of costal margin.

11.18. Convulsive struggles; pink foam and fluid pouring from tracheal tube. No heart palpable. Occasional gasp. Pupils wide.

11.19. No respiration. No heart palpable.

Autopsy at once. No excess fluid in peritoneal cavity. Well marked peristalsis of small gut and cecum; cecum full of gas. Spleen and liver congested. Kidneys apparently normal. No hemorrhages in diaphragm. Lungs do not collapse. Heart large, fills entire pericardial sac. Auricles and ventricles dilated. Heart flabby, large, with no hemorrhages on pericardial or endocardial surfaces. No clot in pulmonary artery or aorta. Lung and trachea excised;

lungs large, erect, heavy, surfaces covered with many small hemorrhages, often confluent. Lower lobes show most hemorrhages; bluish black-red color; upper lobes pink. In trachea pink foam and white foam, alternately in layers. On section, lower lobes extremely juicy, like a wet sponge; not much air, chiefly red fluid with some foam. Upper lobes contain a large amount of white foam, not much fluid; not heavy like lower lobes (Fig. 2).

Of 27 rabbits whose vagi had been divided, 21 showed on autopsy a marked pulmonary edema of the general type described in the preceding protocol. 14 died within 20 minutes of the single intratracheal injection of adrenalin, and of these 10 died within 10 minutes, showing the general symptoms noted above. In only 6 did the autopsy show slight or no edema. Thus in about 78 per cent (21 out of 27) of the animals a single intratracheal injection of a moderate dose of adrenalin caused a marked pulmonary edema.

#### *Control Series with Vagi Intact.*

In this group of sixteen animals the adrenalin was usually injected repeatedly into the trachea, the time interval varying between 4 to 20 minutes. The dosage was usually 0.25 cc. per kilo. In addition to the intratracheal injection a number of the rabbits received intramuscular injections of adrenalin.<sup>5</sup> This series therefore forms a severe control for the group with divided vagi.

The results are briefly as follows: None of the rabbits, in spite of the repeated injections, died within 20 minutes; 3 succumbed after 40 to 45 minutes. The survivors (13) were either killed by medullary puncture 15 to 60 minutes after the first dose of adrenalin or were etherized after 15 minutes and the heart was clamped off. Autopsy revealed in no instance a pulmonary edema of the degree which was found in the series with divided vagi. Pulmonary edema, however, was present to some degree in the lungs of most of the animals; in 6 rabbits the edema was moderate, in the remaining 6 it was slight; one autopsy record was lost.

A comparison of the two series shows clearly that the intratracheal injection of adrenalin exerts a much severer effect in vagotomized

<sup>5</sup> Specimen protocols in tabular form will be found in our previous article, Auer and Gates,<sup>1</sup> pp. 760-761.

than in normal animals. Thus 14 out of 21 vagotomized animals died acutely in 20 minutes after a single intratracheal injection of about 0.25 cc. of adrenalin per kilo, while only 3 out of 16 normal animals succumbed within 40 minutes, although the normal animals received the adrenalin repeatedly. In addition it must be emphasized that the degree of pulmonary edema was much greater in the vagotomized series than in the control series whose vagi were intact.

*Behavior of the Heart.*—In a number of experiments where the blood pressure was recorded, it was observed repeatedly that the blood pressure at one time or another after the adrenalin injection showed sudden, profound drops varying in duration from 10 seconds to 3 minutes associated usually with convulsions after the low pressure level had been attained. Foam in the trachea was noted in some cases, especially those in which the low pressure level was maintained for some time. In Fig. 1, for example, foam first appeared in the trachea after the second series of convulsions following the second abrupt drop of blood pressure.

These abrupt drops of blood pressure were obtained not only in animals with intact vagi, but also in those whose vagi had been sectioned. The cause of these drops was therefore peripheral and probably located in the heart itself. In order to gain information on this point the heart was exposed in a series of seven etherized animals by splitting the sternum and opening the pericardial sac. Usually both pleural sacs were also opened, the respiration being maintained by intratracheal insufflation. In all except one animal the vagi were severed. A specimen protocol will illustrate the observations made and their time relations.

*Rabbit 2.*—Gray female; weight 1,885 gm.

10.20. On pad at low. Ether.

10.46. Operation finished: both vagi divided; cannula in trachea; chest split longitudinally to expose heart. Insufflation of air-ether with remissions; air catheter through tracheal cannula.

Change pad to medium. Heart beats regular, rapid after opening pericardium. Both pleural cavities open; lungs pink, good distension; spontaneous respiration present.

+10.50. 0.47 cc. of adrenalin into tracheal cannula (air catheter temporarily withdrawn), 0.25 cc. per kilo.

10.51. Left ventricle beats half as fast as right ventricle, right auricle, and left auricle. Left ventricular contractions weaker than those of right.

10.54. Both ventricles beat synchronously, following auricles; left ventricle stronger beat than before. No foam in trachea.

11.01. Chambers beat synchronously; good strength.

+11.03½. 0.47 cc. of adrenalin, into trachea.

11.04. Left ventricle half as fast as right ventricle; left auricle very large now; both auricles and right ventricle beat at same rate; left ventricle at half that rate and contractions weak.

11.06. As before, but left ventricle beats more strongly.

11.06½. Left ventricle shows strong and weak beat alternately.

11.08. Left ventricle now beats at same rate as right ventricle; left auricle much smaller now.

Animal killed by clamping the heart. On excising the lungs, the right side is large, full, rounded, with no emphysematous blebs on surfaces; the left side collapses fully. Right lung shows a good amount of foam in the alveoli and large bronchi; the left lung exhibits only slight edema. The surfaces show a moderate number of pin-point hemorrhages. Heart beats vigorously after excision; there are numerous hemorrhages over the left and right ventricles. Left auricle shows extensive hemorrhage on inner surface. Right ventricular endocardium shows a fair number of hemorrhages, only a few in left ventricle. Heart swiftly passes into rigor.

The protocol given above does not, however, show all the gross changes which may be observed in a series of experiments by inspection of the heart. The change of the left ventricular rate to half of that of the right ventricle is usually preceded by a stage in which the left ventricle shows an alternation of a weak and a strong beat. Then the weak beat becomes no longer detectable to the eye and the left ventricle apparently beats at half the right ventricular rate. The left auricle usually beats at the same rate as the right half of the heart, but in one instance its rate was that of the left ventricle—the right half of the heart thus contracted twice to one contraction of the left half. This alteration in the functional activity of the left ventricle was noted in six of the seven experiments. The duration varied from a few seconds to 3 minutes.

In a few instances the entire heart stopped for the length of a beat or two, which accounts for the abrupt blood pressure drops mentioned previously. The dilatation of the left auricle was often tremendous, and made this structure look like a bright red blister. This dilatation is not dependent upon section of the vagi, for a sim-



ilar large dilatation of the left auricle with marked swelling of the aorta and pulmonary artery was also noted in the single experiment of this series in which the vagi were intact.

The pulmonary artery may dilate tremendously after the intratracheal injection of adrenalin; the dilatation develops shortly after the injection and may last 7 minutes. This dilatation is just as marked during diastole of the right ventricle as during its systole. Dilatation of the pulmonary artery was noted in three experiments, not looked for in three, and not seen, though sought for, in one experiment.

The aorta dilated markedly shortly after the adrenalin injection in five experiments and its condition was not observed in two.

In two experiments the adrenalin seemed to exert a locally inhibiting effect upon the muscle of the ventricle. In one instance the upper half only of the right ventricle contracted during systole, the lower half dilating. In this experiment the state of the aorta and pulmonary arteries unfortunately was not observed, but the left auricle dilated enormously and the left ventricle showed the changes in beat already described.

In the second instance the musculature of the left ventricle was affected locally, and with each systole a prominent though small, bright red bulging appeared near the apex. In this animal the aorta and left auricle were strongly dilated; the left ventricle showed the changes in character and rate of beat described previously; the pulmonary artery was not observed. The pulmonary edema obtained in this animal was only slight.

In this series of experiments with double vagotomy and exposure of the heart under artificial respiration none of the animals died acutely, but all were killed after at least 20 minutes had elapsed, by clamping the heart or by medullary puncture. Moreover, the pulmonary edema observed in the excised lungs was only slight in four, and fair to moderate in the other three experiments.

In these experiments the right auricle and right ventricle showed no marked dilatation at any time, nor was any dilatation of the left ventricle observed which could account for the swelling of the left auricle by regurgitation through the mitral valves. On the contrary, the left ventricle was in a state of greater tone with small systolic and diastolic excursions, so that the distension of the left auricle was caused by the inability of this chamber to empty all the blood it received from the lungs into the tonically contracted left ventricle.

Although these experiments yielded interesting information concerning the activity of the heart after the repeated administration of adrenalin in vagotomized animals, the main result is the suggestive absence of any considerable pulmonary edema. This is the more interesting because all the conditions apparently favored the production of pulmonary edema: pressure in the pulmonary veins was surely raised because the dilatation of the left auricle shows that it received more blood than it could handle, so that back pressure resulted; the pressure in the pulmonary artery was also raised as shown by its dilatation; and still further proof of increased pressure in the pulmonary circulation is furnished by the development of hemorrhages during inspection of the lung.

As these experiments were necessarily carried out under artificial respiration, a small series of tests was made to study the effect of artificial respiration upon the production of pulmonary edema when the chest was intact. Four rabbits were utilized; their vagi were divided and intratracheal insufflation started with air-ether in various proportions; in one animal air only was given after the operation had been completed. The adrenalin dose was 0.25 cc. per kilo intratracheally, administered once. In this series one animal died 17 minutes after the injection without any convulsions or foam in the trachea. Death was due to too much ether; autopsy showed pregnancy and only a slight degree of pulmonary edema. The other three animals were killed by medullary puncture 25 minutes after the adrenalin was injected, and the lungs were examined at once. None of them showed a marked degree of pulmonary edema, but some was present in all, especially in the lower lobes.

These results thus indicate that artificial respiration has a restraining effect upon the formation of pulmonary edema, and corroborate a similar observation made by Emerson.<sup>6</sup> How this protection is possibly exerted will be discussed later.

#### *Tracheal Stenosis.*

In another set of experiments the effect of an inspiratory decrease of pressure in the lung alveoli upon the production of pulmonary

<sup>6</sup> Emerson, H., *Arch. Int. Med.*, 1909, iii, 368.

edema in animals with vagi intact was tested. This was accomplished by compressing the trachea with a clamp. The air now entered so slowly during inspiration that a rarefaction of the intrapulmonic air occurred, as was evidenced by the sinking in of the costal margins and of the lower part of the sternum. The lung alveoli thus must exert some suction during inspiration upon the capillaries in their walls, and this should aid in the production of an adrenalin pulmonary edema in normal rabbits (vagi intact).

Fifteen experiments were made, of which ten were controls. The adrenalin animals received 0.3 cc. of adrenalin per kilo intratracheally; after about 2 minutes the trachea was stenosed. About 15 minutes after the injection the animals were killed and the lungs examined at once.

In 5 rabbits where adrenalin had been injected intratracheally and the trachea stenosed, 4 exhibited a good or well marked pulmonary edema; in 1 the edema was slight. The edema, however, was never as great as that seen in vagotomized animals.

In 5 controls, adrenalin was injected in the same dosage, but the trachea was merely exposed, but not constricted. 4 of these rabbits showed slight or no edema, but the fifth exhibited a well marked pulmonary edema. This latter animal, however, was abnormal, for both peritoneal and pleural cavities contained a large amount of clear straw-colored fluid.

In the second set of controls no adrenalin was injected but the trachea was constricted and the animal killed by asphyxia 15 minutes after beginning the stenosis. Of these five animals, all showed either slight or no edema of the lungs. It is seen that stenosis of the trachea and final asphyxia do not suffice in the normal rabbit to bring about pulmonary edema.

This series of experiments thus shows clearly that the cupping action of the lung alveoli on the pulmonary capillaries during inspiration plays a part in the production of adrenalin pulmonary edema.

#### *Distribution of the Pulmonary Edema.*

The edema obtained after vagus section was not uniform in its distribution throughout the lung. Invariably the lower lobes were much juicier and more hemorrhagic than the middle and upper lobes. On section the heavy, lower

lobes resembled sponges soaked to saturation with a pink fluid; squeezing usually yielded little foam but much fluid. The middle and upper lobes showed chiefly white foam with little fluid; occasionally, however, the middle and upper lobes also contained much fluid.

Hemorrhages also were much more extensive over the surfaces of the lower lobes than over the upper lobes (Fig. 2). The outer third of the upper lobes was usually free from hemorrhages.

As a rule, the right and left halves of the lungs were equal in size, but in a number of instances the right side was appreciably larger than the left, and in some of these cases the pulmonary edema was also greater in the larger half.

### *Atropine Series.*

This series of experiments was undertaken in order to test the effect of adrenalin after paralysis of the motor vagus endings. The animals were tracheotomized, the vagi divided, and atropine sulfate was injected in 1 per cent solution about 5 minutes before the adrenalin. In some cases the blood pressure was recorded. The thorax was intact, and no artificial respiration was given. The dose of atropine varied between 1 and 2 mg. per kilo; the amount of adrenalin was always 0.25 cc. per kilo, injected intratracheally. The atropine was injected intramuscularly in the first three animals but in the remaining seven it was administered intratracheally.

Of 3 animals which received atropine intramuscularly, 2 died acutely in 10 to 14 minutes after a single injection of adrenalin showing typical symptoms of pulmonary edema. The other rabbit, however, gave no clinical signs of pulmonary edema within 1 hour, during which he received not one but three doses of adrenalin, each 0.25 cc. per kilo, at 15 to 20 minute intervals. Death occurred after 2½ hours and the lungs exhibited a marked pulmonary edema. This last animal received a larger dose of atropine than the two preceding ones; the first two received 1 and 1.25 mg. respectively per kilo, while the third rabbit was injected with 1.5 mg. per kilo.

In the other seven experiments the atropine was injected intratracheally, the first dose tested being 1.25 mg. per kilo. This amount, in subsequent tests, was increased until 2 mg. per kilo were given. In this series the results were encouraging. 1 animal showed foam and fluid in the trachea, but was in fair condition 35 minutes after the adrenalin injection. The other 6 showed no clinical sign of edema,

the respiration was easy, no râles were palpable or audible, and no foam or fluid was ever seen during the 30 to 40 minutes of observation. All the animals were then killed by medullary puncture and the lungs examined: 5 animals showed slight or no edema, 1 showed moderate edema, and 1 exhibited a marked degree of pulmonary edema. This animal was the one in which foam and fluid issued from the trachea 15 minutes after the adrenalin injection.

Five control experiments without atropine were made with animals from the same lot. Of the 5, 2 died within 10 minutes and the remaining 3 succumbed within 20 minutes after the adrenalin injection. The lungs of all showed a marked degree of pulmonary edema. The differences between lungs of these two series is well brought out by Figs. 2 and 3.

The following table summarizes the results of the atropine series and its controls.

*Atropine Series. Vagi Divided. All Injections Intratracheal.*

No. of animals used.	Atropine per kilo.	Adrenalin per kilo.	Death in 10 min.	Death in 20 min.	Autopsy.
	<i>mg.</i>	<i>cc.</i>			
7	1.25-2.00	0.25	0	0	Slight or no pulmonary edema in 5 animals; moderate pulmonary edema in 1; marked pulmonary edema in 1.
5	None given.	0.25	2	3	Marked pulmonary edema in all.

The protective action of atropine against adrenalin pulmonary edema in normal animals has been noted by Biedl.<sup>7</sup>

#### DISCUSSION.

In the previous pages we have shown that adrenalin pulmonary edema occurs more frequently, in a more severe form, and after a smaller dose in rabbits if the vagi are sectioned previous to the intratracheal administration of the drug, than when the adrenalin is given in the same way to rabbits with vagi intact. It has been pointed

<sup>7</sup> Biedl, A., *Innere Sekretion*, Berlin, 2nd edition, 1913, i, 522.

out that artificial respiration strongly reduces the degree of pulmonary edema after adrenalin when the vagi have been divided, and this result is obtained both with opened and intact chests. Data have been furnished which indicate that after adrenalin administered intratracheally the heart ventricles temporarily deliver unequal amounts of blood with each systole, the right ventricle delivering more blood than the left, shown by the tremendous dilatation of the left auricle and by the marked swelling of the pulmonary artery, which remained distended during diastole as well as systole; it has also been shown that adrenalin produces a temporary incoordination of the heart so that the left ventricle beats at one-half the rate of the right auricle and ventricle. Finally we have demonstrated that atropine markedly reduces the occurrence of pulmonary edema after vagus section and adrenalin.

What bearing do these facts have on the production of pulmonary edema? Leaving aside for the moment the striking adjuvant action of vagus section in the formation of pulmonary edema, it will be noted in the first place that the cardiac changes observed by us after the administration of adrenalin are in full accord with the fundamental postulate of Welch's theory<sup>8</sup> for the production of a general acute pulmonary edema. It will be remembered that this is a disproportion between the ventricular outputs of such a character that the left ventricle expels less blood per systole than the right, thus producing an acute venous hyperemia of the lungs. In our experiments on rabbits whose hearts were exposed for inspection (artificial respiration) we saw this demand fulfilled: shortly after the intratracheal injection of adrenalin the pulmonary artery dilated widely and remained dilated during each diastole of the heart; at the same time the left auricle swelled tremendously and sooner or later the left ventricle showed a rate which was only half that of the right side of the heart, and its amplitude of contraction was small. These facts indicate unquestionably that the right heart is acting vigorously, that the arterial and venous pressures in the lung are

<sup>8</sup> Welch, W. H., *Virchows Arch. path. Anat.*, 1878, lxxii, 375. See also his later presentation in S. J. Meltzer's Harrington Lectures on Edema, *Am. Med.*, 1904, viii, 37 of reprint.

raised, and finally that the left heart is surely expelling less blood per systole than the right.<sup>9</sup>

The physical conditions for the production of pulmonary edema demanded by Welch are therefore present, and yet the pulmonary edema which we obtained in this series with vagi divided, where the heart was exposed under artificial respiration, was slight and even negligible when compared with the tremendous edema which resulted when the same dose of adrenalin was injected into animals (vagi divided) whose thorax was intact and which were breathing in the normal way. Artificial respiration thus seemed to be an inhibitory factor, a supposition which was strengthened by the results of another series of experiments with chest intact, where the vagi were divided and intratracheal air insufflation with rhythmical remissions of the pressure was given throughout. Here again no marked degree of pulmonary edema was observed after the injection of adrenalin, either clinically, or after killing the animal and examining the lungs. Moreover, there are statements in the literature which furnish direct and indirect evidence regarding the effect of artificial respiration on pulmonary edema.

Emerson<sup>6</sup> in a brief note reports that gentle artificial respiration, with or without suction, produces an amelioration of the pulmonary edema in cats, caused by the intravenous injection of massive doses of adrenalin. That artificial respiration may even prevent adrenalin pulmonary edema in rabbits follows directly from a statement of Miller and Matthews<sup>10</sup> that they were never able to obtain pulmonary edema in a considerable number of rabbits by injecting intravenously 0.5 to 2 cc. of 1:1,000 adrenalin. Now, 1 to 2 cc. of adrenalin, intravenously administered, are sure to cause pulmonary edema in a percentage of normal animals provided that the number tested is not too small, and Miller

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<sup>9</sup> While our experiments show only that a rise of pressure undoubtedly exists in the pulmonary circulation, it might be assumed that it is due solely to back pressure from the left auricle. There is, however, good evidence to show that adrenalin contracts the pulmonary blood vessels, and without entering into the question of pulmonary vasomotors, the following observers, who have noted a rise of pressure in the pulmonary artery or a constriction of the pulmonary vessels after adrenalin, may be mentioned: Weber, E., *Arch. Physiol.*, 1910, Suppl., 410; 1912, 383; 1914, 535. Fühner, H., and Starling, E. H., *J. Physiol.*, 1913-14, xlvii, 301. Tribe, E. M., *ibid.*, 1914, xlviii, 154.

<sup>10</sup> Miller, J. L., and Matthews, S. A., *J. Physiol.*, 1909, iv, 370.

and Matthews state that they used a "considerable number." But artificial respiration was necessary in their experiments to record the pressure in the pulmonary artery, and in view of the statements already made regarding the action of this procedure on the production of pulmonary edema, their failure to obtain edema is readily explained. Hallion and Nepper<sup>11</sup> also give as an impression that acute pulmonary edema in rabbits after the intravenous injection of large doses of adrenalin occurred apparently less readily when the thorax was open than when the chest was intact. They are inclined to attribute this action to mechanical conditions of the experiment, circulatory changes in the lung, and they also mention as a factor the positive intraalveolar pressure produced by artificial respiration.

The question now arises how artificial respiration reduces or prevents the pulmonary edema called forth by adrenalin in rabbits.

Artificial respiration does not prevent cardiac changes resulting in an acute congestion of the lungs, for we have observed, as described previously, such cardiac changes in rabbits when the chest was open. Emerson's tentative explanation<sup>12</sup> that the distension of the lung by artificial respiration drives a considerable amount of blood into the left auricle thus relieving the pulmonary congestion, is unsatisfactory even for the conditions theoretically deduced or experimentally observed by him; for he states that the adrenalin causes acute dilatation of the left ventricle with consequent mitral regurgitation, acute congestion of the lungs, and a dilatation and failure of the right heart; edema results due to the back pressure from the left ventricle. On the basis of this conception it is impossible for us to see how the massage of blood by artificial respiration from the lung into an acutely dilated left ventricle with incompetency of the mitral valves, could reduce the back pressure which, according to Emerson, causes the edema. In this connection we may say that we did not observe any acute dilatation of the left ventricle or regurgitation into the left auricle, nor have we seen any marked dilatation of the right ventricle during the cycle of cardiac changes induced by adrenalin. The left ventricle in the rabbit after adrenalin always seemed in a state of greater tone than normal. A few times the entire heart apparently stopped for a few beats in diastole but without any resulting marked dilatation. We are inclined therefore to believe that

<sup>11</sup> Hallion and Nepper, *J. physiol. et path. gén.*, 1911, xiii, 893.

<sup>12</sup> Emerson,<sup>1</sup> p. 370.



artificial respiration with positive pressure does not exert its inhibitory action on pulmonary edema through an action on the heart itself.

There is a factor, however, in the production of pulmonary edema, which is abolished by ordinary artificial respiration, that has received practically no consideration so far as we know. That factor is the aspirating action of the lung alveoli under certain conditions during inspiration. When the diaphragm descends, the negative pressure in the thorax increases, and air enters freely into the alveoli through the trachea and bronchi. The air pressure in the alveoli and consequently upon the capillaries in the alveolar walls remains at atmospheric level. If, however, there is a partial or even complete obstruction in the bronchioles, for example, by contraction of the bronchial muscles, hindering or preventing the entrance of air, then during each inspiration every alveolus connected with such a constricted bronchiole must act like a miniature dry cup, for the pressure in the alveoli and on the capillaries in their walls must decrease as these elastic chambers expand due to the disproportion between intra-alveolar and intrathoracic pressures. If in addition there is a congestion in the pulmonary circulation the passage of a transudate into the alveoli is surely facilitated if not even initiated by this pressure decrease in the alveoli. Experimental support for this view is given by the series of experiments where adrenalin was injected in rabbits whose tracheæ were stenosed.

This conception that the lung alveoli may act like dry cups, has been mentioned as early as 1845 by Mendelsohn.<sup>13</sup> It must be added, however, that this action of the pulmonary air cells occupies but a subsidiary part in Mendelsohn's elaborate development of the thesis that lung hyperemia in general depends upon diminished expansion and contraction of the lungs during respiration. For, according to Mendelsohn, distension of the lungs stretches the pulmonary artery and its capillaries, increasing their volume capacity and thus aspirating blood; any diminution of the expansion and contraction diminishes this aspiration and reduces the pulmonary circulation which now depends solely upon the propulsive power of the right ventricle, so that stasis occurs.<sup>14</sup>

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<sup>13</sup> Mendelsohn, *Arch. physiol. Heilk.*, 1845, iv, 277.

<sup>14</sup> See also Mendelsohn's final publication, *Der Mechanismus der Respiration und Cirkulation, oder das explicierte Wesen der Lungenhyperämien*, Berlin, 1845.

Is there any evidence that such a constriction of the bronchioles occurs after the injection of adrenalin? Since the publication of Kaplan's<sup>15</sup> observation that adrenalin relieves bronchial asthma, most of the investigators describe a relaxation of the bronchial muscles as the result of an adrenalin injection,<sup>16</sup> yet bronchial constriction by the same substance has also been noted. Golla and Symes<sup>17</sup> with a new method of artificial respiration obtained constriction of the bronchioles in decerebrate cats and rabbits after adrenalin unless the bronchioles were initially constricted by other drugs, under which condition a relaxation was obtained. One of us, in some unpublished work, has also observed oncometrically a definite decrease in the amplitude of the lungs in rabbits after the intravenous injection of adrenalin, and it has already been stated that we have often seen a distension of the excised rabbit lung after adrenalin which was not accounted for by the degree of pulmonary edema present.

There is thus sufficient experimental proof that adrenalin may cause a constriction of the bronchial muscles and it is evident that as soon as this occurs the cupping action of the alveoli during inspiration can take place, thus aiding the passage of fluid from the gorged capillaries into the alveoli.

On the basis of this action of adrenalin, the protective action of artificial respiration may be explained; the positive pressure which artificial respiration produces in the lung reduces or overcomes the bronchial constriction due to the adrenalin and thus prevents the intraalveolar pressure from becoming negative during inspiration. Consequently the cupping action on the pulmonary capillaries does not occur. Thus one link in the chain of conditions which are more or less necessary for the production of pulmonary edema is broken, and edema is then produced with greater difficulty and to a less extent.

The protective action of atropine against pulmonary edema from adrenalin after vagus section may also be explained by paralysis of the bronchomotor endings of the vagus, and the consequent inability

<sup>15</sup> Kaplan, D. M., *Med. News*, 1905, lxxxvi, 871.

<sup>16</sup> Januschke, H., and Pollak, L., *Arch. exp. Path. u. Pharm.*, 1911, lxvi, 206-214. Trendelenburg, P., *ibid.*, 1912, lxix, 104. Jackson, D. E., *J. Pharm. and Exp. Therap.*, 1912-13, iv, 74, 291; 1913-14, v, 509. Bachr, G., and Pick, E. P., *Arch. exp. Path. u. Pharm.*, 1913, lxxiv, 62, 71. Dixon, W. E., and Ransom, F., *J. Physiol.*, 1912-13, xlv, 413.

<sup>17</sup> Golla, F. L., and Symes, W. L., *J. Pharm. and Exp. Therap.*, 1913-14, v, 88.

of adrenalin to bring about constriction of the bronchioles, thus preventing the aspirating action of the alveoli. This is in accord with the prevailing view concerning the point of attack of both atropine and adrenalin. It must be mentioned, however, that Golla and Symes<sup>18</sup> report that 10 mg. of atropine in a rabbit failed to relieve the bronchoconstriction caused by adrenalin. It is of course obvious that atropine may also affect the heart or the lung vessels and endothelia in such a way as to prevent or reduce pulmonary edema. But as we have no direct experimental evidence of our own we shall not enter into a discussion of these possibilities.

The aspirating action of the lung alveoli during coexisting pulmonary congestion also explains why the edema is always greater in the lower lobes than in the middle and upper lobes. This is due to the fact that the negative pressure is greater in the lower lobes than in the others. Experimental evidence for this is furnished by the work of Meltzer<sup>19</sup> and Meltzer and Auer,<sup>20</sup> which indicates that the negative pressure in the chest is not the same at all levels but is greatest in the lower portion of the thoracic cavity. If a bronchiolar constriction occurs during a marked hyperemia of the lungs it follows that the cupping action of the alveoli during inspiration must be most effective where the negative pressure variation is greatest, provided that the hyperemia and endothelial permeability are the same throughout the lung.

We come now to a consideration of the remarkable accelerating effect which vagus section exerts upon the production of adrenalin pulmonary edema, and it may be stated at once that we cannot offer a full analysis because the experimental test of the various possibilities is incomplete. It may be said, however, that this difference in action is apparently a quantitative one, for pulmonary edema can usually be obtained in the normal rabbit provided that sufficient adrenalin is administered without causing cardiac death. The optimum state for the occurrence of a strong pulmonary edema develops more slowly in a rabbit with vagi intact than in one with vagi sectioned. This suggests that the structure primarily responsible for

<sup>18</sup> Golla and Symes,<sup>17</sup> p. 93.

<sup>19</sup> Meltzer, S. J., *J. Physiol.*, 1892, xiii, 218.

<sup>20</sup> Meltzer, S. J., and Auer, J., *J. Exp. Med.*, 1910, xii, 34.

the fulminant onset of edema is the heart. By vagus section the heart is deprived of important regulating influences, and incoordination of the two sides of the heart perhaps then results more readily under stress; that such an incoordination does take place promptly when the vagi are divided and adrenalin is administered, we have already shown. Our experiments on the action of adrenalin on the fully innervated exposed heart of the rabbit are too few to permit any conclusion. The electrocardiographic studies on the action of adrenalin in the dog made by Kahn<sup>21</sup> and by Rothberger and Winterberg<sup>22</sup> which showed no incoordination of the heart after the vagi were sectioned (Kahn), but elicited changes in the form of waves very similar to those obtained by faradic stimulation of the accelerators (Rothberger and Winterberg), tend to support our view because the dog after vagus section tolerates large doses of adrenalin without developing pulmonary edema.

Another action which vagus section may facilitate is the production of bronchial constriction by adrenalin. After vagus section the bronchial muscles are relaxed and thus in the optimum state for contraction as demanded by Golla and Symes. The adjuvant action of bronchial constriction in the production of pulmonary edema has already been discussed.

Vagus section not only facilitates the formation of pulmonary edema after adrenalin, but it is an indispensable preliminary for obtaining pulmonary edema in experimental hydremic plethora.

Kraus<sup>23</sup> reports that the infusion of large quantities of salt solution in cats and rabbits only caused pulmonary edema when the vagi were sectioned. He also observed that adrenalin aided in the production of this edema; that open or closed thorax exerted no influence; and that atropine did not prevent this form of edema. Kraus assumes tentatively that section of centripetal vagus fibers, which govern reflexly the lung vasomotors, causes a disturbance in the regulation of the blood supply to the lung leading indirectly to edema.

Although Kraus' experiments and our own were carried out under widely different conditions so that a comparison cannot be made,

<sup>21</sup> Kahn, R. H., *Arch. ges. Physiol.*, 1909, cxxix, 379.

<sup>22</sup> Rothberger, J., and Winterberg, H., *Arch. ges. Physiol.*, 1910, cxxxv, 531.

<sup>23</sup> Kraus, F., *Z. exp. Path. u. Therap.*, 1913, xiv, 402.

it is nevertheless highly suggestive to note that in both sets of experiments vagus section exerted a profound influence. The nature of this influence cannot be stated with certainty at the present time.

In the preceding pages we have made no attempt to give a complete presentation of the problem of pulmonary edema but have limited ourselves largely to those facts which follow directly from our experiments. Necessarily, therefore, numbers of factors have been touched only lightly or not at all in the discussion.

#### SUMMARY.

The intratracheal injection of one moderate dose of adrenalin in rabbits whose vagi are divided produces a marked pulmonary edema in a large percentage of cases. The same dose in normal animals causes only slight effects.

Artificial respiration greatly reduces the production of pulmonary edema in vagotomized rabbits after adrenalin.

As adrenalin can exert a bronchoconstrictor effect, evidence is submitted to show that the aspirating action of the lung alveoli under this condition apparently plays an important part in the production of adrenalin pulmonary edema. On the basis of this mechanism the protective action of artificial respiration is explained.

Stenosis of the trachea facilitates the production of adrenalin pulmonary edema in rabbits whose vagi are intact.

The intratracheal injection of adrenalin in vagotomized rabbits produces a temporary incoordination between the heart ventricles, visible on inspection, so that the left ventricle beats apparently half as fast as the right, causing hyperemia of the lungs and hemorrhages.

Atropine injected intratracheally in vagotomized rabbits exerts a protective action against adrenalin pulmonary edema.

#### EXPLANATION OF PLATES.

##### PLATE 14.

FIG. 1. Blood pressure record. Rabbit 3; black female. Weight 1,980 gm. Both vagi were divided. The time is marked in 4 second intervals. The time line is also the zero pressure level. 0.5 cc. of adrenalin (0.25 cc. per kilo) was injected into the tracheal glass cannula. Note the three abrupt drops in the blood pres-

sure with prompt recovery. The low level (25 to 50 mm. of mercury) was maintained for 20 to 60 seconds, and convulsions appeared during this time. The typical foam of pulmonary edema appeared in the trachea after the second fall, and pink fluid during the terminal descent of the blood pressure. The terminal fall was slowed by a partial clot. These abrupt pressure drops are possibly caused by a complete cardiac stoppage such as was observed when the heart was exposed for inspection.

PLATE 15.

Figs. 2 and 3 show the protective effect of atropine. In both rabbits the vagi were divided and both received 0.25 cc. of adrenalin per kilo intratracheally. Rabbit 5, however, was injected with 1.75 mg. of atropine sulfate per kilo intratracheally before the adrenalin was administered.

FIG. 2. Rabbit 4, adrenalin alone. Typical pulmonary edema developed, with death in 18 minutes. The lungs were large, heavy, erect, hemorrhagic, with foam and fluid in the tracheal cannula.

PLATE 16.

FIG. 3. Rabbit 5, which received atropine before the adrenalin, showed no symptoms of pulmonary edema, and was killed after 45 minutes. The lungs collapsed practically normally, but the surfaces were peppered with small hemorrhages. The trachea showed no foam or fluid.





FIG. 1.



220<sup>6</sup>



FIG. 2.

(Auer and Gates: Adrenalin pulmonary edema.)

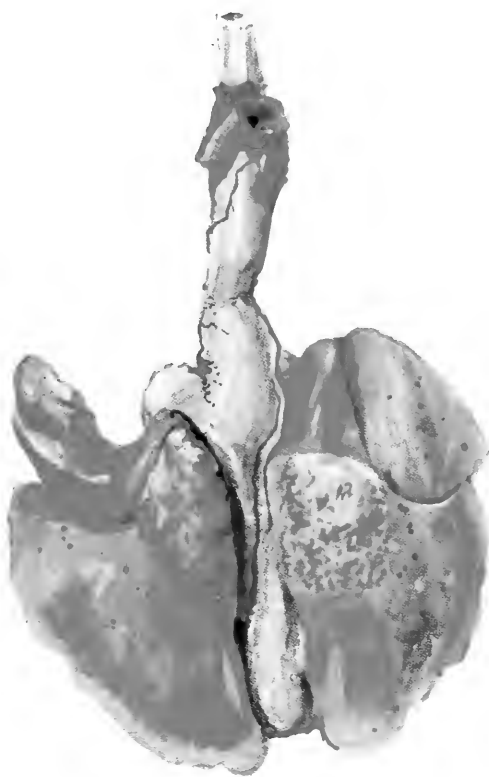


FIG. 3.

(Auer and Gates: Adrenalin pulmonary edema.)



## THE EFFECTS OF EXPERIMENTAL PLETHORA ON BLOOD PRODUCTION.

By OSWALD H. ROBERTSON, M.D.

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Our present knowledge concerning normal blood production indicates that the bone marrow functions at a constant rate supplying new red corpuscles to replace those lost daily in the process of normal blood destruction. Since an extra loss of blood causes greater activity of the bone marrow,<sup>1</sup> it has seemed possible that were the constantly occurring normal loss compensated for artificially there would be no necessity for the production of new cells, and consequently a lessened activity of the bone marrow might result. In an attempt to bring about this condition, rabbits have been made plethoric by repeated small transfusions of blood and the effect of the procedure on the bone marrow has been observed.

Some work has already been done in this direction. Hess,<sup>2</sup> with the purpose of determining the effect of plethora on the heart, gave rabbits repeated large transfusions. At the end of 2 months of plethora the rabbits were killed. In examining the bone marrow, he claimed to have found evidence of a markedly diminished activity. The erythroblasts and myelocytes were decreased in number, and there was a fibrous hyperplasia present. Itami<sup>3</sup> gives a more detailed account of these findings, agreeing with Hess in his conclusions. Boycott and Douglas<sup>4</sup> carried on a similar series of experiments but failed entirely to confirm Hess's and Itami's results, which seem to have been obtained in only a few animals.

<sup>1</sup> The term "bone marrow activity," as used in this paper, refers only to the erythropoietic function of the marrow.

<sup>2</sup> Hess, R., *Deutsch. Arch. klin. Med.*, 1909, xcv, 482.

<sup>3</sup> Itami, S., *Folia hematol.*, 1908, vi, 425.

<sup>4</sup> Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909, xiii, 414.

The criteria which these two sets of investigators used as evidence of depressed bone marrow activity, that is, marked histological changes in the marrow, may account to some extent for their contradictory results, since the red marrow of even normal rabbits varies much in its morphology as indicative of blood production and also in its distribution.

In the present work, use has been made of an indicator which may be thought to represent more nearly the actual functional variations in the marrow taken as a whole; namely, the number of reticulated red cells in the circulating blood. In addition, the number of these cells in the bone marrow itself has been determined. In normal animals, reticulated red corpuscles are present in the blood in fairly constant numbers. There is good evidence that increased activity of the bone marrow is accompanied by an increase in the percentage of these cells, and that the percentage roughly parallels the degree of hyperfunction.<sup>5,6</sup> *A priori*, one would suppose that if the activity of the bone marrow were lessened, a drop in the reticulated cell count would result.

### *Method of Producing Plethora.*

The method of producing the plethora has already been described<sup>7</sup> but will be briefly given here. Rabbits were used. Each recipient was provided with three to six donors of the same hemolytic group. Transfusions of 10 cc. of blood were made daily. The donors were used in rotation, so that each one lost at most only 10 cc. every 3 days. In this way the production of any considerable anemia in the donor rabbits was avoided. In some of these rabbits, the blood loss was made up so promptly that the hemoglobin percentage did not vary throughout the period of the bleedings; in others it dropped slightly. The blood for transfusion was obtained from the donor by cardiac aspiration into a syringe containing 1 cc. of a 1 per cent solution of sodium citrate in normal salt solution. This small amount of citrate, when well mixed with 10 cc. of blood, was sufficient to prevent coagulation for the few minutes required to introduce the blood into the marginal ear vein of the recipient. The recipient rabbits for the most part weighed from 1,500 to 1,800 gm. All were young rabbits. Both sexes were employed.

<sup>5</sup> Lee, R. I., Minot, G. R., and Vincent, B., *J. Am. Med. Assn.*, 1916, lxvii, 719.

<sup>6</sup> Vogel, K. M., and McCurdy, U. F., *Arch. Int. Med.*, 1913, xii, 707.

<sup>7</sup> Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665.

The Sahli hemoglobinometer was used for determining the degree of plethora. The inaccuracies of this method are recognized, but dependable readings were obtained by diluting for the color comparison after the test mixture had stood exactly 5 minutes. In some of the animals, the red cells were counted and the color index was estimated.

### *Method of Counting Reticulated Cells.*

The following method for counting the reticulated cells was found to be preferable to those previously described. A saturated solution of brilliant cresyl blue was made up in normal salt solution. This was kept as a stock solution. When a count was to be made, a small quantity of it was diluted 80 times<sup>8</sup> with normal salt solution and mixed with blood in a pipette for counting white cells in the proportion of one part of blood to twenty parts of cresyl blue solution. The mixture was shaken in the pipette for 5 minutes. The cells were thus equally distributed as well as stained. They were counted at once in fresh preparations, which were sealed with vaseline to prevent disturbances due to drying. At least 1,000 red cells were counted at each test. When the number of reticulated cells was less than 1 in 1,000, 10,000 red cells were counted. In the latter case, only the first 1,000 were counted individually, the field being the unit of count for the remaining 9,000.

For several days before transfusions were begun, the number of reticulated cells was determined daily in those rabbits destined to be recipients.

In a large number of normal rabbits examined, the reticulated cells were found to vary for the most part between 10 and 20 per 1,000 erythrocytes. Rarely they were as many as 30 or fewer than 5 per 1,000. Only two animals showed less than 5 per 1,000, one having a count of 3 and the other of 4. The variation in the individual from day to day may be slight or, relatively speaking, considerable.

<sup>8</sup> Since doing this work, a second saturated solution of cresyl blue has been made up, using a different stock of the dye, which went into solution to a considerably greater extent than the first. The result was that a 1:80 dilution of this saturated solution was much too strong a staining fluid. It was found necessary to dilute to 180 for satisfactory staining. It is apparent, therefore, that each saturated solution of cresyl blue has to be tested beforehand for its optimum staining dilution. This is a very simple matter and needs to be done only once.

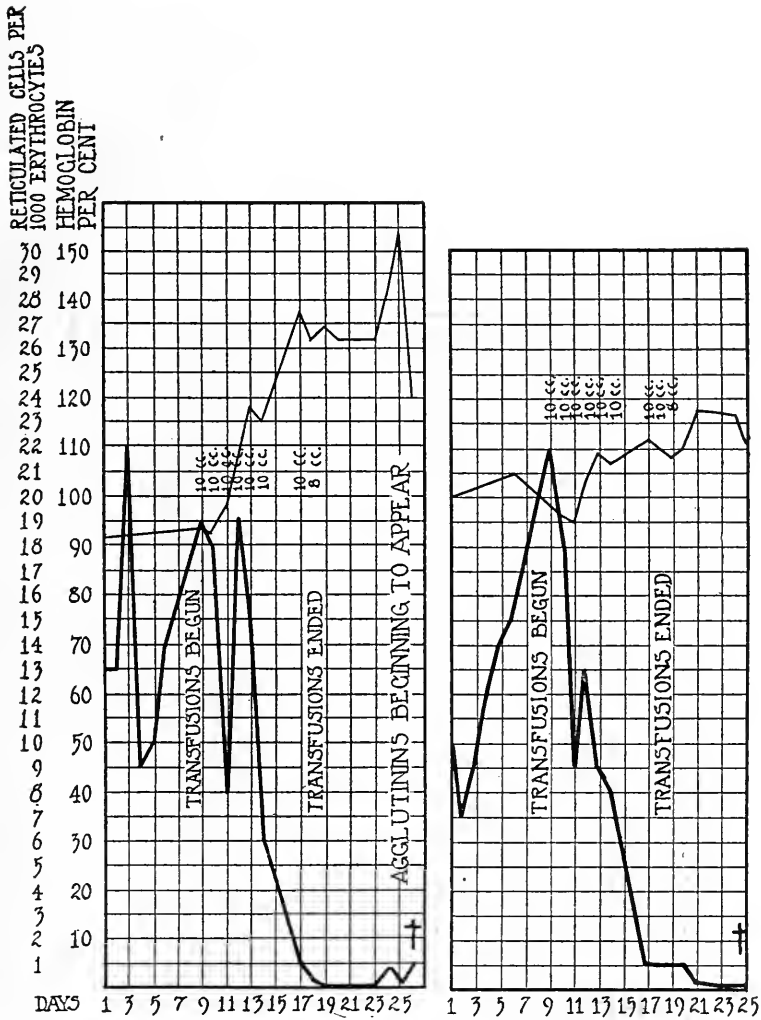
*Production of Plethora.*

Sixteen rabbits were rendered plethoric by the method described. The rate at which the hemoglobin per cent increased in different animals varied considerably. In some it rose rapidly. In others it increased slowly. The degree of plethora ultimately attained likewise varied much. An amount of blood which in one animal resulted in a rise of fifty points in the hemoglobin per cent, produced in another individual of the same size a rise of only thirty points. For this reason some of the animals were transfused more often than others. Even so it was often found impossible to force up the hemoglobin per cent in these refractory individuals to a height easily reached in other animals. The shortest period of transfusion was 9 days, during which the animal was given seven transfusions; the longest, 20 days with nineteen transfusions. As a rule, the hemoglobin per cent rose from a normal of 80 to 90 per cent to 140 to 150 per cent, at which point it remained fairly constant despite the continued introduction of blood.

No notable change in the percentage of reticulated cells occurred until after several transfusions. Then, as plethora became well defined, the number of reticulated cells in the circulating blood began to diminish (Text-figs. 1 and 2). This decrease in number was sometimes rapid and soon became marked. Often the reticulated cells practically disappeared. Counts below 1 in 10,000 were frequent (Text-fig. 1), and sometimes a search through a whole slide would reveal none. The charts of Text-figs. 1 and 2 will illustrate the change that occurred in eight of the sixteen recipients. In five others, the drop in reticulated cells was less marked, though definite. They decreased to about 1 in 1,000. The degree of plethora was fully as great in these animals as in the afore mentioned ones, and no reason has been found for the differences observed.

Anomalous results were obtained in three rabbits. One showed only a slight drop in the reticulated red cells (Text-fig. 3). In the remaining two, these cells failed to decrease at all. One had a purulent pneumonia, with some anemia, despite the transfusions. The other developed an increased number of reticulated cells for which no cause was ever found.

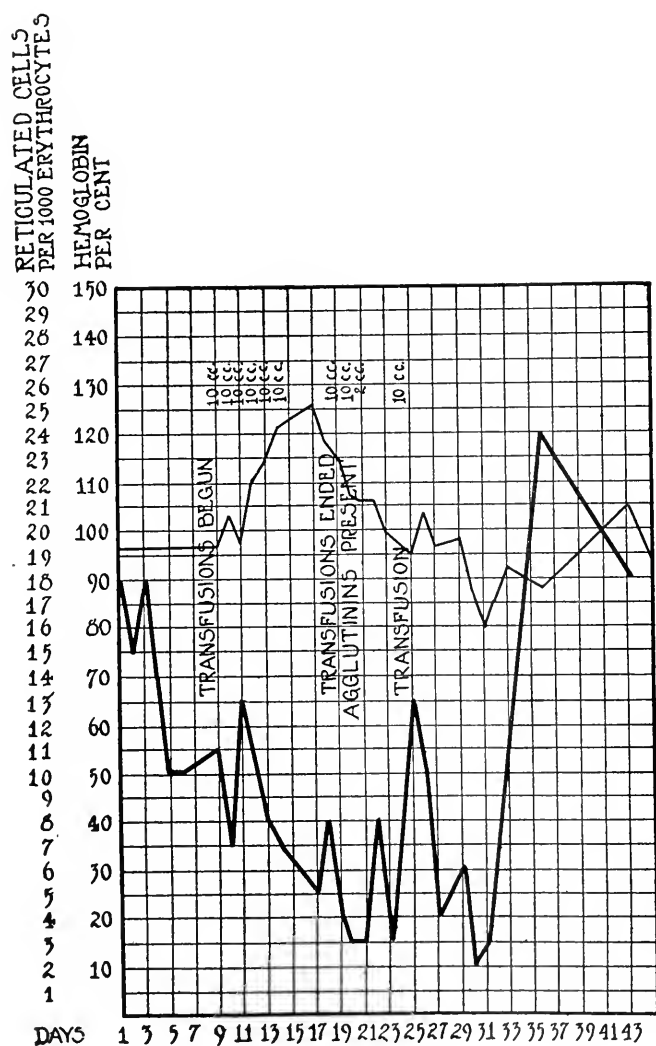




TEXT-FIG. 1.

TEXT-FIG. 2.

— Reticulated cells.  
 — Hemoglobin.



TEXT-FIG. 3.

It is evident from these results that a diminution in the number of reticulated cells in the circulation follows almost constantly the production of an artificial plethora. A possible influence of sodium citrate in causing this change has been ruled out by observations in five rabbits which received daily injections of sodium citrate alone, in some cases twice as much being given as in the rabbits made plethoric. The number of reticulated cells did not decrease in these animals.

#### *Examination of the Bone Marrow.*

The bone marrow from five of the eight rabbits in which the reticulated cells dropped markedly was examined for its content of these cells. The following procedure was employed.

Red marrow from the upper and lower ends of the femur was taken into a mixture of equal parts of Locke's solution containing  $\frac{1}{4}$  per cent of gelatin and an isotonic watery solution of sodium citrate. In this mixture clotting does not occur, and even the most delicate cells are well preserved. The gross appearance of the marrow was normal except for a congestion such as was present in all the other organs. A small portion of it was teased on a slide in a drop of the Locke's-citrate mixture and a count made at once from the preparation. Then, with a view to washing out the cells of the marrow, the needle of a syringe was thrust into it here and there, and gelatin-Locke's solution injected under considerable pressure. In this way a large part of the substance of the marrow was washed out. The washings were then centrifuged and counts made on the sedimented cells according to the technique used for the peripheral blood. To obtain a figure for purposes of comparison, an average was taken from the counts on the teased specimen and the washings.

The average number of reticulated cells in the bone marrow of the five plethoric animals was 14 per 1,000 red cells, the highest number 22 and the lowest 5 per 1,000. In striking contrast are the figures obtained in the examination of five normal rabbits. These showed an average of 320 per 1,000 red cells, the highest 540 and the lowest 160 per 1,000.

#### *Anemia Following Plethora.*

In certain of the plethoric rabbits there occurred, after many transfusions, a sudden marked drop in hemoglobin. In one instance (Text-fig. 4), despite daily transfusions of 10 cc. of blood, the hemo-

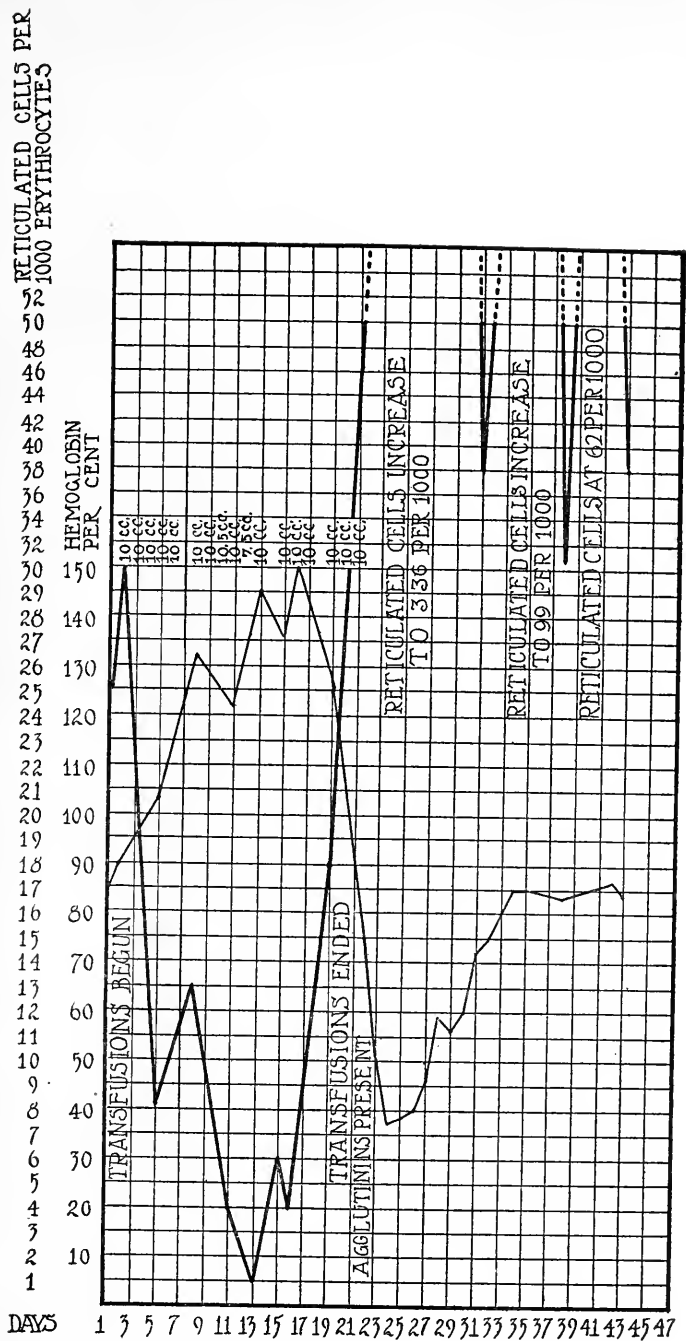
globin fell in 6 days from 150 per cent to 75 per cent. Transfusions were discontinued in such animals, and there rapidly ensued a severe anemia. This was observed in three rabbits. Charts of two of them are shown in Text-figs. 4 and 5. The cause of the rapid transition from plethora to anemia has not yet been determined, but it is significant that isoagglutinins for the donors' blood developed in all three rabbits at about the time when the hemoglobin first began to fall. The simplest explanation would seem to be that, following a number of transfusions, a lytic process develops against the strange cells, and they are rapidly destroyed. This results not merely in a return of the hemoglobin to normal but in an anemia, because of the peculiar condition of affairs indicated by the drop in reticulated red cells during the plethora. During it the bone marrow activity sinks far below normal, whereas blood destruction is maintained at the normal rate at least. Thus, little by little, as the animals' own cells are destroyed and not replaced, the bulk of the circulating blood comes to be strange blood liable to destruction when the lytic principle develops. The extent to which the blood cells proper to the recipient have been destroyed and replaced by alien cells is suddenly revealed through the destruction of this alien blood.

The anomalous behavior of one rabbit, whose chart is shown in Text-fig. 3, strongly supports this explanation of the phenomenon. In this animal there came at length a drop in the hemoglobin, but instead of progressing to an anemia, as in the three rabbits described above, it sank to a point only slightly below normal. The failure of anemia to develop here would seem to be due to the fact that during the plethora, blood production had been maintained at almost its normal rate as indicated by the lack of any but a slight diminution in the number of circulating reticulated cells.

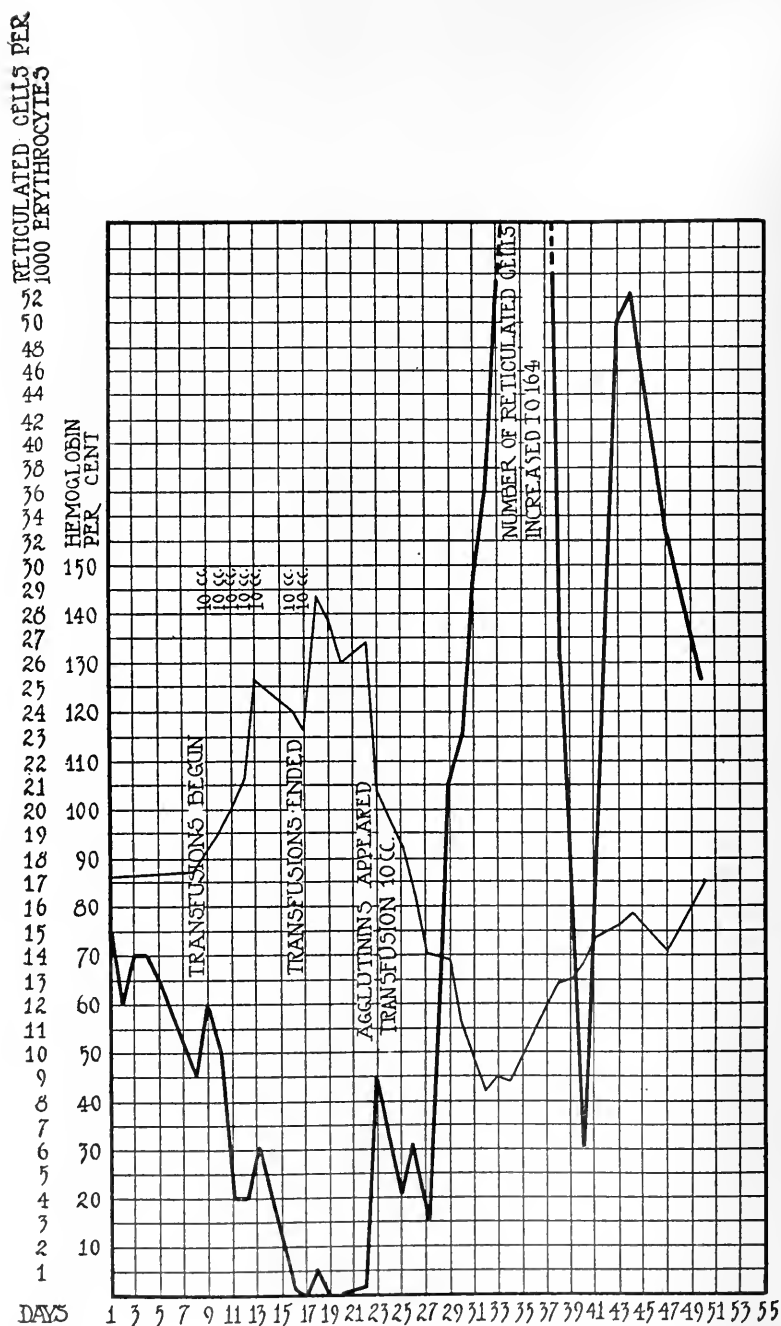
The recovery from the anemia in the three rabbits just mentioned was exceedingly rapid. A similar rapid recovery was noted by Muir and M'Nee<sup>9</sup> and Itami,<sup>10</sup> following experimental hemolytic anemias.

<sup>9</sup> Muir, R., and M'Nee, J. W., *J. Path. and Bacteriol.*, 1911-12, xvi, 410.

<sup>10</sup> Itami, *Arch. exp. Path. u. Pharm.*, 1910, lxii, 104.



TEXT-FIG. 4.



TEXT-FIG. 5.

*Increased Bone Marrow Activity During Subsidence of the Plethora.*

The beginning hemoglobin drop in those rabbits which developed an anemia during or after transfusion was marked in each case by a prompt rise in the number of reticulated cells. This increase was rapid, and in two cases the number had risen above normal by the time the hemoglobin had descended to its original level (Text-fig. 4). Then, as the hemoglobin fell further and anemia developed, the reticulated cells continued to increase with great rapidity. The largest number was observed during the early period of regeneration. One rabbit showed a maximum of 594 per 1,000 at this time. They gradually diminished as the animal recovered from the anemia but were still increased when the hemoglobin reached normal again and remained fairly numerous for some time.

Attention is called to the fact that the number of reticulated cells began to increase soon after the hemoglobin started to fall from the plethora level and long before it had reached normal. A further study of this phenomenon was considered worth while. It seemed not unlikely that stimulation of the bone marrow at this time might be brought about by the greatly increased quantity of destroyed blood present. Accordingly an attempt was made to determine this possibility by injecting rabbits intravenously with laked blood. The rabbits were first rendered anemic<sup>11</sup> by bleeding, and the injections of blood were made at different stages of recovery. Some of the rabbits were treated when very anemic; others had practically reached normal before treatment was begun. In order to simulate as nearly as possible conditions of blood destruction occurring *in vivo*, the laked blood was injected at 1 hour or 2 hour intervals for periods of 1 to 3 days. Relatively large quantities were given without apparent ill effect. At no time during the course of the experiment was any increase in the number of reticulated cells noted, nor was other evidence obtained of increased bone marrow activity.

It was then found that simple blood removal from a plethoric animal by bleeding was sufficient to cause a marked bone marrow

<sup>11</sup> The reason for producing a preliminary anemia in these rabbits is that the experiment was performed originally in an attempt to explain on experimental grounds the cause of the remission in pernicious anemia.

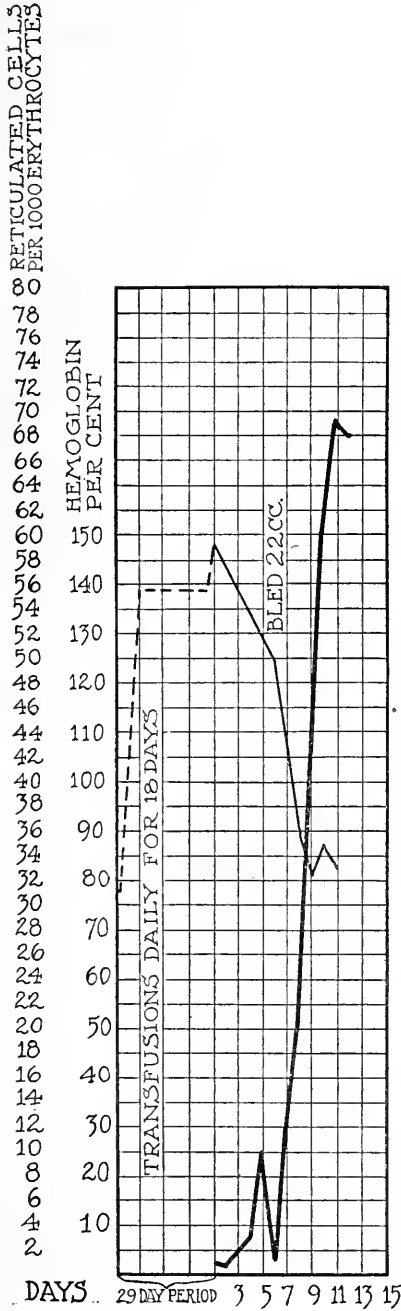
stimulation. Rabbits were made plethoric in the usual way and kept in this condition until the reticulated cells were much diminished. They were then bled a quantity calculated to bring their hemoglobin per cent back to almost normal. An immediate rise in the number of reticulated cells resulted. The normal number, it will be recalled, is 5 to 20 per 1,000 red cells. In one animal treated as above described, they increased to 69 per 1,000 (Text-fig. 6); in another to 80. A third rabbit showed even a greater increase, 108 per 1,000 (Text-fig. 7), but unfortunately the hemoglobin in this case fell after bleeding to slightly below normal. With a fourth animal, the increase was definite, but less marked. In Text-fig. 6 it will be noted that the fall in hemoglobin following bleeding extended over a period of several days, and that the reticulated cells had increased markedly some time before the hemoglobin reached its normal level. Text-fig. 7 shows an instance in which the drop in hemoglobin as a result of bleeding was later followed by a rise above normal. This secondary rise in hemoglobin and the high reticulated cell count were maintained for some days.

Although these experiments are not sufficiently complete to permit one to draw definite conclusions, yet the results would suggest that the increased bone marrow activity accompanying the initial drop of hemoglobin in the plethoric rabbits is due to some functional disturbance of the circulation, resulting from the rapid removal of the plethora. It seems not improbable that this may be a temporary relative oxygen deficiency explainable on the basis that during the period of plethora the organism had in some way adapted itself to a blood of greater oxygen-carrying power. Certainly the blood loss may be thought of as having resulted in a relative anemia.

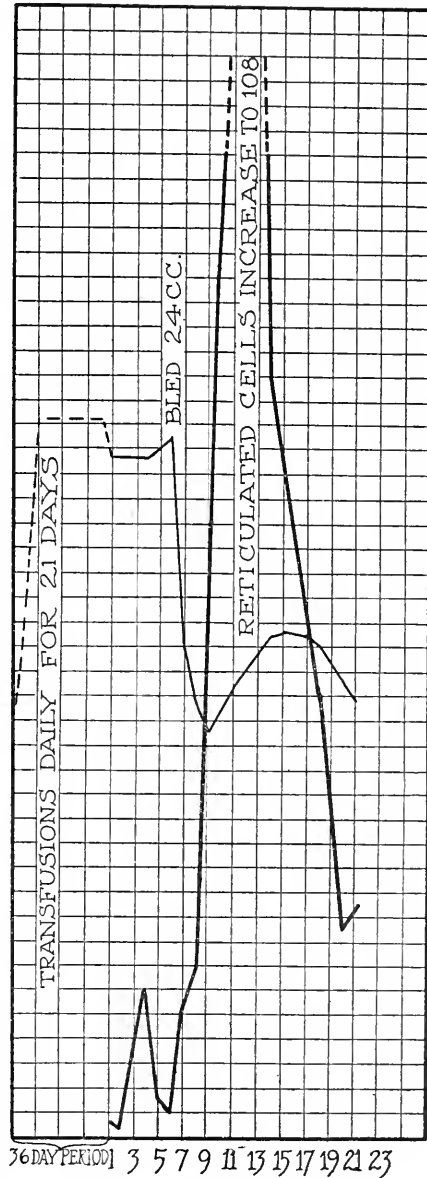
#### *Color Index.*

Observations on the color index in those plethoric rabbits developing anemia revealed striking changes. The normal color index for rabbits is 0.65 to 0.75. During regeneration it was greatly increased, usually to 1.0 or even to 1.08. This finding is of interest particularly in its relation to the accompanying greatly increased percentage of reticulated cells in the blood. It would indicate that reticulation is





TEXT-FIG. 6.



TEXT-FIG. 7.

not necessarily associated with a deficiency in the hemoglobin content of the cells and that this association in the blood of secondary anemia is largely fortuitous. In two of the present instances, the reticulated cells numbered 34 per cent and 60 per cent respectively of the total number of red cells, yet the color index was increased 50 per cent over the normal. Even allowing for the somewhat increased size of the reticulated cells, it does not seem possible that they contained less hemoglobin per unit of cell substance than the accompanying more normal red cells. In fresh films they were observed to be notably well colored.

#### *Transfused Reticulated Cells.*

In the work as thus far discussed, a possible source of error has not been considered; namely, that of the reticulated cells introduced with the transfused blood. The number of these may, and indeed sometimes must be, considerable, because the bone marrow of the animals furnishing the blood has been stimulated by repeated bleeding. After many transfusions one might expect the recipients to show high counts of reticulated cells. As a matter of fact the reverse is the case. What becomes of the reticulated cells introduced, whether they perhaps mature into non-reticulated corpuscles or instead are destroyed, remains to be determined. As bearing on the point, it should be mentioned that the microcytes of secondary anemia due to hemorrhage are largely derived from fragmentation of reticulated cells, and that in plethoric animals the fragmentation of red blood cells is much increased.<sup>7</sup>

No observations were made on the effect of plethora on the number of white cells or platelets.

#### *Clinical Bearing.*

The effect of experimental plethora on the bone marrow of rabbits has a direct bearing on certain unfavorable results which may occur after transfusions in human beings. Clinicians have observed that some cases of pernicious anemia receiving transfusion show no stimulation, but instead unmistakable signs of bone marrow depression.

Vogel and McCurdy<sup>6</sup> were the first to report a systematic study of the reticulated cells in pernicious anemia patients with transfusion. In several of their cases, a decided drop in the per cent of reticulated cells occurred following transfusion. They attributed this to the diluting effect of the newly introduced blood, which had a much lower content of reticulated cells. These patients did badly.

At the Massachusetts General Hospital, Minot and Lee<sup>12</sup> have recently observed the effect of transfusion on the per cent of reticulated cells in a number of cases of pernicious anemia. In certain of their patients, a very marked diminution in the reticulated cells occurred following transfusion. This was accompanied by a leukopenia, a reduction in the number of platelets, and in some cases purpura as well, all of which went to form the picture of general bone marrow depression. As was to be expected, there was no increase in the red corpuscles after transfusion in these patients, other than that referable to the alien blood introduced. In their cases, the amount of blood transfused did not exceed 600 cc. and in one case it was only 300 cc. No such effects as those described above were observed after transfusions of less than 300 cc.

From the results described in the present paper, one may draw tentative conclusions as regards such instances. In pernicious, as in any form of anemia, oxygen deficiency resulting from blood loss doubtless constitutes the stimulus for increased blood production. It is conceivable that in severe conditions, a stage is reached where the bone marrow becomes so exhausted that there is danger of its failure to respond any longer to this stimulus; in other words, the stimulus of oxygen deficiency has grown relatively less effective and may at any time become insufficient. The introduction of a large quantity of blood into the circulation has inevitably the effect of reducing oxygen lack. The sudden lowering of stimulus thus brought about may result in a diminished activity of the bone marrow. The inference is clear that in pernicious anemia with a sluggish bone marrow as shown by the count of reticulated cells, small transfusions are preferable to large ones.

<sup>12</sup> Dr. Minot and Dr. Lee have very kindly allowed me to use these data, which they have not yet published.

## SUMMARY.

With the purpose of determining whether a diminished activity of the bone marrow could be brought about experimentally, plethora was produced in rabbits by means of repeated small transfusions of blood. Counts of the number of reticulated red cells in the circulating blood were made during the course of the experiments as an index to changes in the activity of the bone marrow.

With the development of plethora, the number of reticulated cells in the blood decreased. In the majority of the plethoric animals, this diminution was extreme, and in some instances, reticulated cells practically disappeared from the blood. A comparison of the red bone marrow of these animals with that of normal controls revealed a marked reduction in the content of reticulated cells.

After a number of transfusions, there occurred in some of the plethoric rabbits a sudden and marked drop in hemoglobin. The hemoglobin continued to fall until a severe grade of anemia was reached. This was followed by an extremely rapid regeneration accompanied by a striking rise in color index. During regeneration, the reticulated cells were enormously increased in number.

Taken together, these facts show that the bone marrow is markedly influenced by plethora. The diminished number of reticulated cells observed, both in the circulating blood and in the marrow, would make it appear that a decided decrease in blood production occurs. The reduction in the number of these cells cannot be due to changes in the constitution of the red cells put out by the bone marrow, as a result of an increased quantity of hemoglobin in the body, because during regeneration from the above mentioned anemia, when the color index was very high, reticulated cells were still present in large numbers. That the activity of the bone marrow does actually diminish during plethora is further evidenced by the occurrence of the anemia. The most reasonable explanation of this phenomenon is that the recipient develops an immunity against the blood of the donors, which results in the destruction of the strange cells that are in circulation. In keeping with this conception is the appearance of isoagglutinins for the donors' red cells in the blood of the recipient, at about the time of the beginning fall in hemoglobin. The occurrence of anemia as a result of the destruction of the alien blood only would seem to be

due to the circumstance that, during the period of plethora, blood production is greatly diminished; as a consequence, the blood cells proper to the recipient are gradually reduced in number and replaced by alien cells until the latter come to constitute the bulk of the animal's blood.

In those rabbits developing anemia, the initial drop of hemoglobin from the plethoric level to the normal was constantly accompanied by a marked rise in the number of reticulated cells. This brought up a subsidiary problem for study. With the idea that the stimulation of the bone marrow might be due to the presence of an increased quantity of broken down blood, rabbits were injected intravenously with large amounts of laked blood cells. The procedure had no evident effect on the blood picture. It was then found that simple blood removal from a plethoric animal which brought back the hemoglobin to the normal level, or even to a point somewhat above, sufficed to cause a marked increase in the number of reticulated cells. Although these findings are not conclusive, they suggest an explanation for the increased bone marrow activity accompanying the initial drop of hemoglobin in the plethoric rabbits; namely, that the organism had in some way adapted itself during the period of plethora to the presence of a greater amount of blood and that the result of blood loss in such an organism was a relative but not absolute anemia.

The finding that the activity of the bone marrow can be depressed by the introduction of a large quantity of blood into the circulation accounts for the diminished bone marrow activity which sometimes occurs after transfusion in pernicious anemia. In such cases there is a marked drop in the number of reticulated cells and other evidence of bone marrow depression; the patient shows no benefit from transfusion or may grow rapidly worse. The cause of this depression is best explained on the basis that in severe instances of the disease where exhaustion of the bone marrow is imminent, the stimulus of the anemia is only just sufficient to keep the marrow functioning. A sudden lowering of this stimulus is brought about by the introduction of a large quantity of blood into the circulation, and the result is a fall in the activity of the bone marrow. It follows from this that in pernicious anemia with a feebly reacting bone marrow as indicated by the number of reticulated red cells, small transfusions are preferable to large ones.



## EXPERIMENTAL STUDY OF ORGANIZATION IN LOBAR PNEUMONIA.

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PLATES 17 TO 19.

(Received for publication, March 30, 1917.)

Healing of injured and intensely inflamed vascular tissues is characterized by the formation of granulation tissue. A striking exception to this is the perfect healing of the lung in most cases of lobar pneumonia. Here, complete resolution with removal of the exudate and regeneration of the injured lung result. Occasionally, however, the exudate persists, and, as elsewhere, it is dealt with by an ingrowth of capillaries and connective tissue cells.

The factors determining the result in the inflamed lung are little understood and the explanations offered for the occurrence of unresolved lobar pneumonia are as inconclusive as those for the perfect healing of the lung. Ziegler (1) suggested that the retention of the exudate and its organization in unresolved lobar pneumonia probably depended upon recurring exudates, eventually requiring organization for their removal. Von Kahlden (2) considered an excessive formation and retention of fibrin the probable cause. Kohn (3) believed that organization of the exudate occurred as a result of irritation of the interlobular and pleural connective tissue. Köster (4) thought that blocking of the lymph channels in the involved area was responsible. Marchand (5) considered alcoholism a predisposing factor, and Heschl (6) reported its greater frequency in emaciated individuals. Amburger (7) advocated syphilis as the cause. Another conception was that of Corrigan (8) who described it as a special disease analogous to cirrhosis of the liver.

In 1903 Flexner (9) found that consolidated lung in the stage of red hepatization autolyzed imperfectly, whereas in the stage of gray hepatization it autolyzed rapidly and perfectly. He concluded that in unresolved pneumonia because of some disproportion between the leukocytes and other constituents, or other cause unknown, the inflammatory exudate failed to autolyze perfectly, could not be absorbed, and hence underwent organization.

A few years later Opie (10) in the course of a study of leukocytic enzymes noted that when a mixture of dog serum, leukocytes, and fibrin were incubated, autolysis of the leukocytes and digestion of the fibrin were inhibited, whereas when no serum was present, the leukocytes autolyzed readily and the fibrin was completely digested.

In 1914, in association with Winternitz (11), a study of the circulation in the pneumonic lung was made. It was observed that in the later stages of lobar pneumonia, the circulation through the consolidated portion is extremely poor. This was strikingly shown in animals stained with trypan blue. Two sets of experiments were undertaken. In one series, rabbits were injected intravenously with trypan blue, and, following the injection of the dye, the animals were given an intrabronchial injection of pneumococci. The trypan blue was again introduced intravenously during the progress of the pneumonia. In the other series of animals, the first procedure was the injection of pneumococci intrabronchially, and 40 or more hours later the intravenous injections of trypan blue were begun. In the animals receiving trypan blue first, and during the course of the pneumonia, the consolidated portions of the lung at autopsy were intensely blue, whereas in those animals in which the trypan blue was injected only in the later stages of the pneumonia, it was found that but very little of the dye penetrated the consolidated portions. It was inferred that little or no serum reaches the exudate in the alveoli in the stage of gray hepatization, and it was suggested that if this were true, autolysis of the leukocytes and digestion of fibrin in the lung in the late stages of pneumonia are not inhibited and resolution therefore occurs.

Not only do the experiments mentioned above suggest that the absence of the serum from the exudate may be a determining factor in bringing about resolution, but they likewise suggest that the persistence of the exudate in unresolved pneumonia may depend upon the presence in the exudate of sufficient serum to prevent the autolysis of the leukocytes and digestion of the fibrin. If this is true, persistence of the exudate and its organization, in lobar pneumonia, might be expected to develop in those cases in which the circulation in the lung, in the later stages of the process, was good enough to permit a considerable amount of serum to reach the exudate in the alveoli.

The experiments to be reported in this paper were undertaken to determine the significance of the presence of serum in the exudate, in the late stages of pneumonia. The method of injecting the serum depended upon an observation made in the study mentioned above (11). It was found that although very little of the trypan blue,



when injected intravenously, penetrated the lung in the later stage of pneumonia, the exudate offered no barrier to its penetration when it was injected intrabronchially.

The experiments were carried out in dogs because of this animal's resistance to the pneumococcus. Twenty-five animals in all were injected. In nineteen lobar pneumonia was produced with a Group I pneumococcus, according to the technique of Lamar and Meltzer (12). In each case, before the injection of the pneumococci, an x-ray was taken to show the position of the catheter. Seven of the animals were kept as controls, without further treatment. In the other twelve, sterile dog blood serum was injected into the same lobe that received the pneumococci once a day for 4 to 7 days, beginning 48 to 72 hours after the intrabronchial injection of pneumococci. On each occasion x-rays were taken to make sure of the proper position of the catheter.

In addition to the nineteen animals mentioned above, six normal dogs were injected intrabronchially, once a day for 4 to 7 days, with corresponding amounts of sterile dog blood serum.

All the animals were killed in from 9 to 18 days after the first intrabronchial injection. The results are shown in Tables I to III.

TABLE I.

Dog No.	Pneumo- cocci per kilo intra- bron- chially.	Serum per kilo intra- bronchially. Daily, 4 to 7 injections.	Length of life after pneumo- coccus in- jections.  <i>days</i>	Lesion organization.		Lung culture.
				In 1/6 of lobe or over.	Few small areas.	
1	2.5	2.5	9	+		Sterile.
2	3	3	18	+		"
3	4	4	18	+		"
4	3.5	3.5	17	+		"
5	3.5	3.5	17	+		83 colonies of Gram- negative bacilli.
6	3	3	15		+	
7	3.5	3.5	17		+	
8	3.5	3.5	17		+	
9	2.8	2.8	15	—	—	
10	2.5	2.5	15	—	—	
11	3.5	3.5	17	—	—	
12	3.3	3.3	18	—	—	

TABLE II.

Dog No.	Pneumococci per kilo intrabronchially.	Length of life after pneumococcus injections.	Lesion organization.	
			In 1/6 of lobe or over.	Few small areas.
	<i>cc.</i>	<i>days</i>		
13	3.8	18	—	—
14	2.2	15	—	—
15	3.5	17	—	—
16	3.5	17	—	—
17	4.3	18	—	+
18	2.25	9	—	+
19	4	15	—	+

TABLE III.

Dog No.	Serum per kilo intrabronchially. Daily, 4 to 7 injections.	Length of life after first injection of serum.	Lesion organization.	
			In 1/6 of lobe or over.	Few small areas.
	<i>cc.</i>	<i>days</i>		
20	4.6	15	—	—
21	3.3	15	—	—
22	4.16	13	—	—
23	4.5	13	—	—
24	3.5	15	—	—
25	3.5	15	—	—

## ILLUSTRATIVE PROTOCOL.

*Dog 1.*—Weight 12,000 gm.

May 22, 1916, 9.20 p.m. Catheter 17 F inserted into bronchus. X-ray taken (catheter in bronchus to right lower lobe). 30 cc. of 29 hour culture of Group I pneumococcus in broth injected through catheter.

May 25-30. 30 cc. of sterile dog blood serum injected daily through catheter in right lower lobe. X-rays taken before each injection.

May 31, 4 p.m. Animal etherized.

*Autopsy.*—Performed at once. Right lower lobe much more voluminous than normal. Large, solid masses palpable. On section numerous small and larger solid, heavy, airless, consolidated areas are made out, involving one-half of lobe. One area in center the size of small walnut; one near periphery the size of a large walnut. Other areas smaller. These solid patches have a smooth surface and have a grayish red appearance. In branches of the bronchus of this lobe there are firm grayish white plugs, in some places partially, in others completely occluding

the lumen. Histological examination shows the majority of the alveoli in the consolidated portion partially filled with wandering cells (large mononuclear and polymorphonuclear), varying amounts of fibrin, and numerous fibroblasts, in many places associated with fresh capillaries. The process is strikingly shown in the bronchioles where the fibroblasts and fresh capillaries invading the retained exudate are very prominent.

Film preparations of the lung culture taken at the time of autopsy (five loops of expressed tissue juice) on blood agar showed no growth at the end of 3 days.

It will be seen from Table I that in the majority of animals receiving repeated injections of the serum into the pneumonic lung, complete resolution did not occur. In most of these, 9 to 18 days after the production of pneumonia, areas of consolidation in one-sixth or over of a lobe, were present, and microscopically the exudate in the bronchioles and alveoli of the consolidated portions showed extensive organization. In several of the animals the areas of organizing pneumonia were smaller. (Figs. 1 to 5.)

Cultures taken from the lung, in which areas of consolidation were present in one-sixth or more of a lobe, showed no growth in four of the five cases. In the fifth a number of colonies of a Gram-negative bacillus were obtained.

In the majority of dogs with pneumonia not treated with serum, complete resolution occurred (Table II). In two of the three animals in which complete resolution did not occur, no macroscopic areas were observed at the time of autopsy, but a study of the sections showed a small number of alveoli containing exudate undergoing organization. In the third animal there were about twelve pin-head to lentil-sized areas. Most of these showed microscopically an exudate of polymorphonuclear leukocytes, mononuclear cells, scattered red blood cells, and little fibrin. The areas showing organization of the exudate were no more extensive than in the two mentioned above.

In the animals receiving repeated doses of serum alone (Table III) no areas of consolidation were found at autopsy, and microscopically the lungs showed no exudate of any kind.

#### CONCLUSION.

The experiments reported above give evidence that in unresolved lobar pneumonia, the persistence of the exudate, followed by organization, depends upon the presence of serum in the exudate.

I wish to acknowledge my indebtedness to Dr. T. Scholz, of the X-ray Department, for his cooperation, and to Dr. W. G. MacCallum, for helpful suggestions.

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#### EXPLANATION OF PLATES.

##### PLATE 17.

FIG. 1. Bronchiole showing persistent exudate undergoing organization, with attachment to the wall in three places. Dog 2, intrabronchial injection of pneumococci, followed by five daily injections of serum. Killed 18 days after onset of pneumonia. Magnification about 260.

FIG. 2. Alveoli and bronchioles containing persistent exudate undergoing organization. Dog 3, intrabronchial injection of pneumococci, followed by five daily injections of serum. Killed 18 days after onset of pneumonia. Magnification about 260.

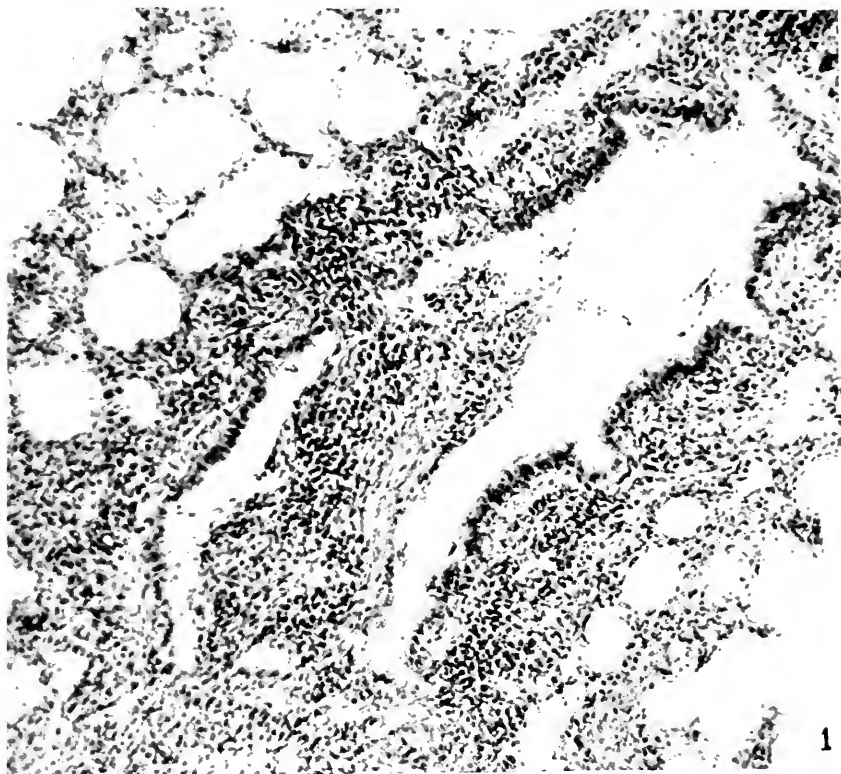
##### PLATE 18.

FIG. 3. Alveoli and bronchioles containing persistent exudate undergoing organization, showing extension of process through the pores of Kohn. Dog 3. Magnification about 260.

FIG. 4. High power of portion of the same section as Fig. 1 (Dog 2), showing attachment of exudate to bronchiole wall and ingrowth of fibroblasts. Magnification about 800.

##### PLATE 19.

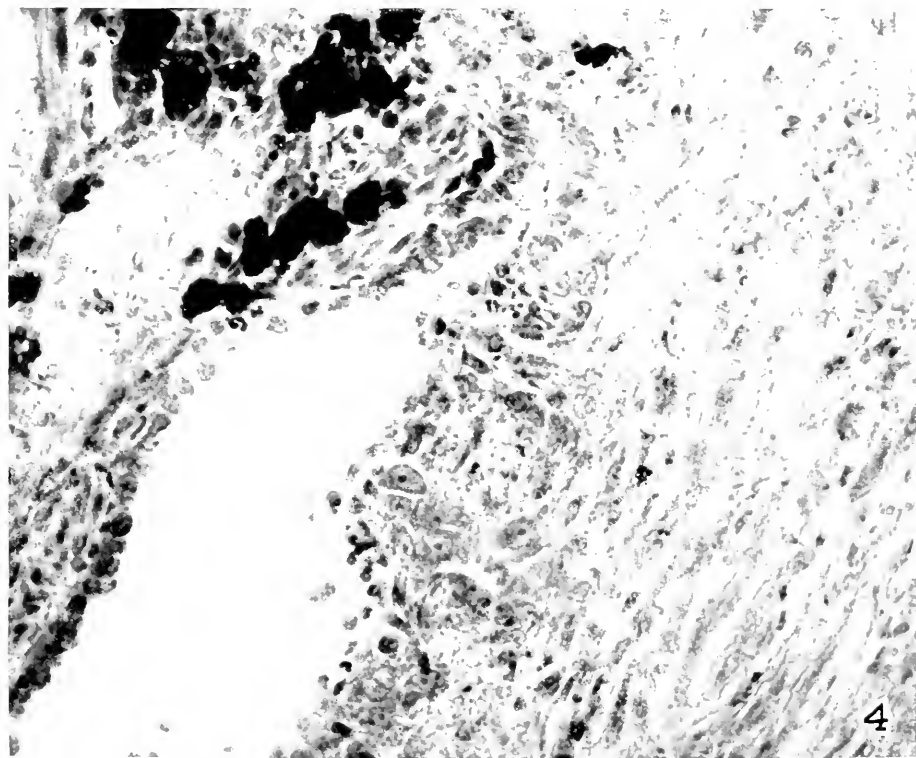
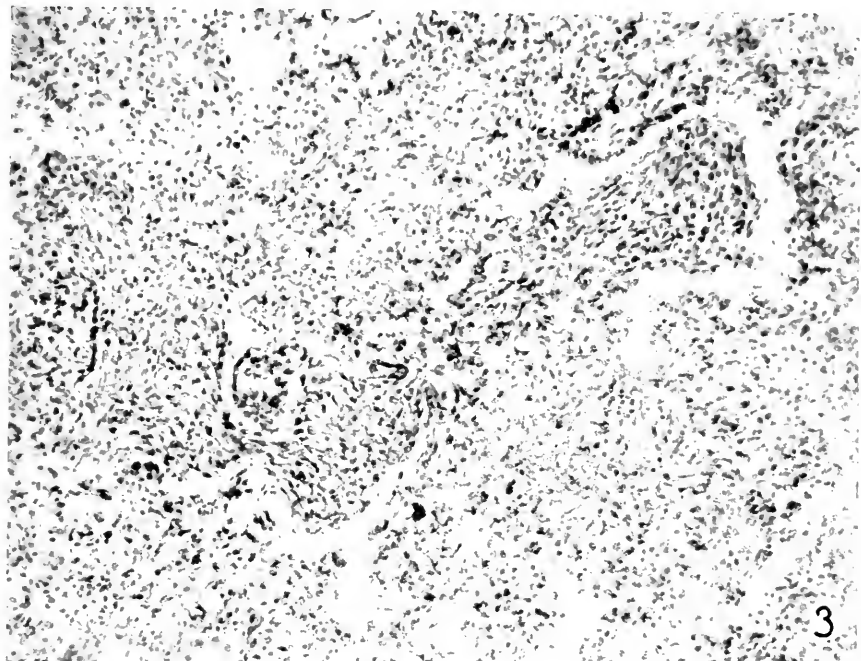
FIG. 5. Another portion of same section as Fig. 1 (Dog 2), showing attachment of exudate to wall and ingrowth of fibroblasts and capillary. Magnification about 800.



(Kline: Organization in lobar pneumonia.)



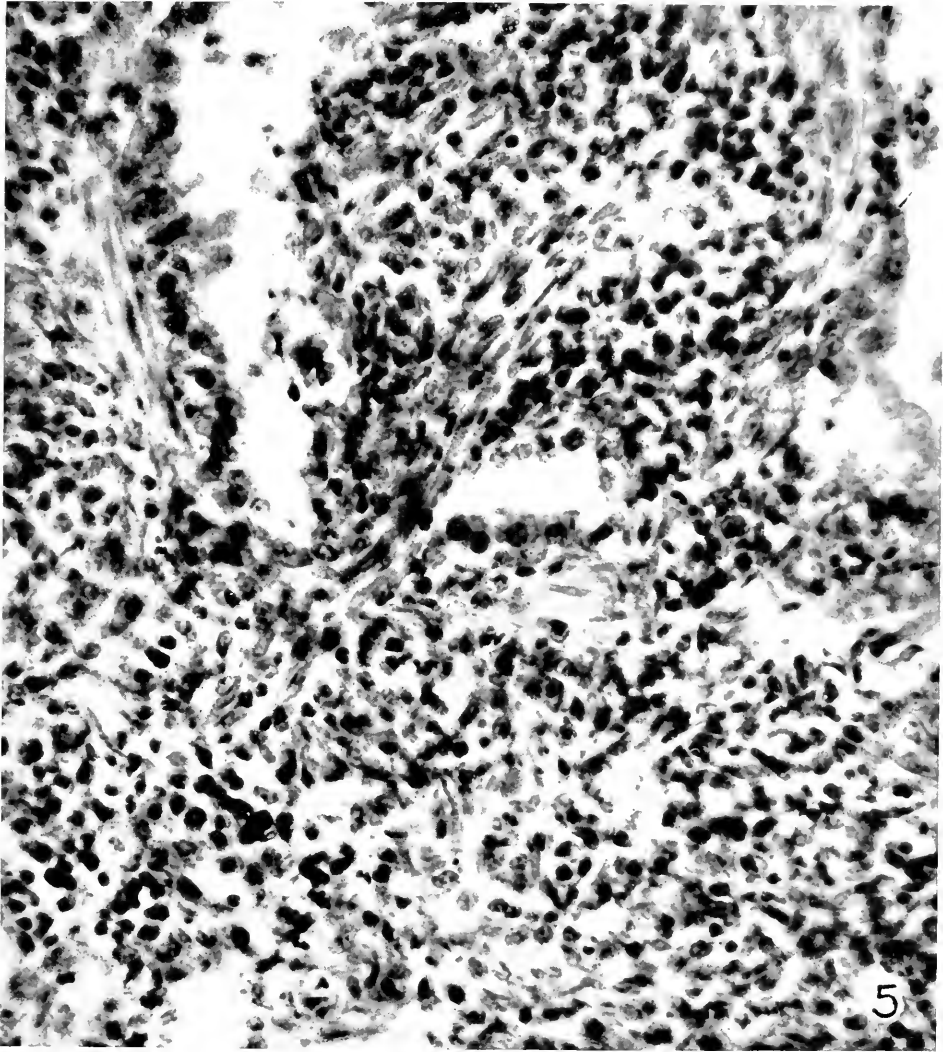
2-4



(Kline: Organization in lobar pneumonia.)







(Kline: Organization in lobar pneumonia.)



# THE GROWTH OF THE OVARIAN FOLLICLE OF THE GUINEA PIG UNDER NORMAL AND PATHOLOGICAL CONDITIONS.

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As the ovary seems to be a favorable organ for the quantitative estimation of cell growth, we undertook to analyze the growth energy of the granulosa cells in the ovaries of normal guinea pigs and also to study the growth energy of these cells under certain pathological conditions.

## *Method.*

The growth energy was estimated by the determination of the percentage of mitoses in granulosa cells in the follicles of the ovaries of normal guinea pigs. A large number of cells was counted and the number of mitoses ascertained, thus making possible a comparison of the proliferative power of the various follicles by the relative percentage of mitoses.

The follicles were classified according to size into three groups, large, medium, and small, whose average measurements were: large, 788 by 611  $\mu$ ; medium, 506 by 450  $\mu$ ; small, 215 by 130  $\mu$ . These measurements are from the inner border of the theca interna on one side to the same point on the other side; *i.e.*, the space lined by the theca interna.

One other type of follicle was studied in normal ovaries; *viz.*, the mature type whose characteristics have been described in earlier papers by Loeb.<sup>1</sup> These follicles are on an average somewhat larger than the ordinary large follicle. There is relatively much more

<sup>1</sup> Loeb, L., *J. Morphol.*, 1911, xxii, 37; *Virchows Arch. path. Anat.*, 1911, ccvi, 278; *Zentr. Physiol.*, 1910-11, xxiv, 203.

cytoplasm in the cells which in hematoxylin- and eosin-stained sections is pink and gives the follicle a much pinker appearance than others in the same ovary.

Measurements of large, medium, and small follicles follow and are of the space lined by the theca interna.

*Large Follicles.*

Long diameter, between	1,076 and	550 $\mu$ .
Short " "	830 "	377 $\mu$ .
Average long diameter,	788 $\mu$ .	
" short "	611 $\mu$ .	

*Diameter of Follicular Cavity.*

Long diameter, between	973 and	434 $\mu$ .
Short " "	726 "	256 $\mu$ .
Average long diameter,	747 $\mu$ .	
" short "	502 $\mu$ .	

*Medium Follicles.*

Long diameter, between	650 and	408 $\mu$ .
Short " "	615 "	315 $\mu$ .
Average long diameter,	506 $\mu$ .	
" short "	450 $\mu$ .	

*Diameter of Follicular Cavity.*

Long diameter, between	575 and	169 $\mu$ .
Short " "	457 "	92 $\mu$ .
Average long diameter,	347 $\mu$ .	
" short "	101 $\mu$ .	

*Small Follicles.*

Long diameter, between	318 and	77 $\mu$ .
Short " "	227 "	77 $\mu$ .
Average long diameter,	215 $\mu$ .	
" short "	130 $\mu$ .	

*Diameter of Follicular Cavity.*

Long diameter, between	188 and	23 $\mu$ .
Short " "	108 "	29 $\mu$ .
Average long diameter,	80 $\mu$ .	
" short "	69 $\mu$ .	

Several of each of the preceding types of follicles, usually three, were studied in each ovary. In order to count the cells and mitoses we studied the follicle in serial sections with a  $\frac{1}{12}$  oil immersion objective and a No. 8 ocular upon whose distal surface two parallel lines were ruled. This method presents some difficulties, one constant source of error being the confusion which results from focusing on the cells at different levels. Such errors are uniform throughout and although they depreciate the value of the estimation of the absolute number of cells in a follicle of a given size to some extent, their influence is practically nil from a comparative standpoint, the error being the same in all cases.

#### *Growth of the Normal Follicle.*

The protocol of Guinea Pig 1 gives the results obtained in the three sizes of follicles approximately the same as the average figures based on all the follicles studied (Table I). The two ovaries of the animal are numbered 1 and 2. It is shown here that there is a distinct difference in the percentage of mitoses of the different types of follicles. The medium follicles have the highest percentage of mitoses and therefore the greatest growth energy, the large ones the lowest, and the small follicles have a proliferative power which slightly exceeds that of the large type, but is considerably less than that of the medium follicle. This fact holds good in either ovary alone as well as the combined figure of both ovaries.

Table II shows the injurious effect of extirpation of corpora lutea by burning and the effect of slight postmortem change on the percentage of mitoses.

This difference in the growth energy of the various types of follicles is constant, whether we consider the follicle of one or two ovaries of the same animal, if the number of cells counted is sufficiently large, and furthermore, approximately the same relative difference in the percentage of mitoses was seen in all the normal ovaries examined.

Small follicles have relatively more variability in the percentage of mitoses than medium or large follicles. Various sized follicles and mature follicles studied in the different ovaries have been grouped and tabulated separately.

Table II gives a summary of the number of granulosa cells and mitoses counted with the percentage of mitoses in the large follicles of the ovaries of two normal guinea pigs, and also the ovaries of two guinea pigs in which the follicles were abnormal.

TABLE I.  
*Protocol of Guinea Pig 1.*

Follicles in Ovary 1.				Follicles in Ovary 2.			
Follicle.	No. of cells.	No. of mitoses.	Percentage of mitoses.	Follicle.	No. of cells.	No. of mitoses.	Percentage of mitoses.
Large follicles.							
No. 1	2,009	19	0.94	No. 1	2,604	8	0.30
" 2	3,550	14	0.39	" 2	3,125	21	0.67
" 3	2,390	25	1.0	" 3	2,349	23	0.97
" 4	2,122	28	1.3				
" 5	2,294	19	0.83				
Total.....	12,365	105	0.84	Total....	8,078	52	0.64
Medium follicles.							
No. 1	1,431	19	1.3	No. 1	2,617	36	1.3
" 2	2,685	20	0.74	" 2	2,076	25	1.2
" 3	2,437	27	1.1	" 3	2,173	15	0.68
Total.....	6,553	66	1.0	Total....	6,866	76	1.1
Small follicles.							
No. 1	749	5	0.66	No. 1	846	3	0.35
" 2	492	5	1.0	" 2	1,441	17	1.1
" 3	681	7	1.0	" 3	747	4	0.53
Total.....	1,922	17	0.88	Total....	3,034	24	0.78

*Large Follicles.*—In Table II the figures for four different guinea pigs are given. The figures given for Guinea Pigs 1 and 2 are compiled from a study of normal follicles and represent the true growth energy of large follicles, whereas the percentage of mitoses in the granulosa cells of Guinea Pigs 3 and 4 has been lowered in one case by burning out of the corpora lutea and in the other by postmortem change.

To summarize, the growth energy of eighteen large follicles has been studied. In normal ovaries 23,954 cells have been counted having a percentage of mitoses of 0.73. In abnormal ovaries 25,344 cells have been counted with a percentage of mitoses of 0.16.

TABLE II.

*Percentage of Mitoses in Large Follicles.*

Guinea pig No.	Ovary.	No. of cells.	No. of mitoses.	Percentage of mitoses.	Remarks.
Normal follicles.					
2		3,511	19	per cent 0.54	Heat 21 days before death. 7 days after ovulation.
1	1	12,365	105	0.84	
	2	8,078	52	0.64	
Total.....		23,954	176	0.67* 0.73†	
Pathological follicles.					
3		8,417	12	0.14	Corpora lutea burned out of ovary. Found dead. Ovaries taken out several hours after death.
4	1	8,813	0	0.0	
	2	8,114	29	0.35	
Total.....		25,344	41	0.16* 0.16†	

\* This figure represents the average obtained from the percentage of the respective ovaries.

† This figure represents the percentage of mitoses for the total of all the cells counted in that particular type of follicle, the total being compiled from the figures of all the follicles counted in the various animals.

*Medium Follicles.*—The figures of medium follicles are shown in Table III. The percentage of mitoses in all these follicles is 1 or more, with the exception of Guinea Pig 3 which is the animal from whose ovaries the corpora lutea had been burned out. Doubtless the low percentage of mitoses here is due to the heating of the ovary in the process of burning out the corpora lutea.

To summarize, in normal medium follicles (Guinea Pig 3 is excluded) 80,625 cells have been counted, having a percentage of mitoses of 1.2.

TABLE III.

*Percentage of Mitoses in Medium Follicles.*

Guinea pig No.	Ovary.	No. of cells.	No. of mitoses.	Percentage of mitoses.	Remarks.
				<i>per cent</i>	
5	1	13,924	147	1.0	10 days after birth of young.
	2	10,990	160	1.4	
6	1	5,823	76	1.3	Near the end of pregnancy.
	2	6,771	96	1.4	
7	1	4,828	55	1.1	Ovulation had just taken place.
	2	5,086	60	1.1	
2	1	9,428	128	1.3	Heat 21 days before removal of ovaries.
	2	10,356	131	1.2	
1	1	6,553	66	1.0	7 days after ovulation.
	2	6,866	76	1.1	
3		4,464	21	0.47	Corpora lutea burned out of ovary.
Total.....		80,625	995	1.1* 1.2†	Total does not include Guinea Pig 3.

\* Average percentage of mitoses.

† Absolute percentage of mitoses.

*Small Follicles.*—Table IV shows that there is relatively greater variability in the percentage of mitoses of small, than either large or medium follicles. The growth energy of small follicles in various stages of cavity formation was compared, but no constant differences were found. Here again the ovaries of Guinea Pig 4, in which there has been slight postmortem change, showed a very low percentage of mitoses and their figures together with those of Guinea Pig 3, from whose ovaries the corpora lutea were burned out, are not included in the total.

To summarize, in normal small follicles 89,545 cells have been counted, having a percentage of mitoses of 0.66.

*Mature Follicles.*—The growth energy of six mature follicles in the ovaries of two guinea pigs was studied (Table V). In all, 24,445



TABLE IV.

*Percentage of Mitoses in Small Follicles.*

Guinea pig No.	Ovary.	No. of cells.	No. of mitoses.	Percentage of mitoses.	Remarks.
				<i>per cent</i>	
5	1	49,520	305	0.61	10 days after parturition.
	2	10,241	25	0.24	
6	1	6,693	84	1.20	Near the end of pregnancy.
	2	1,713	12	0.70	
2	1	6,084	22	0.36	Heat last observed 21 days before death.
	2	2,432	13	0.53	Ovulation had just taken place.
7	1	3,831	60	1.56	
	2	4,075	30	0.73	
1	1	1,922	17	0.88	7 days after ovulation.
	2	3,034	24	0.78	
3		3,929	28	0.71	Corpora lutea burned out of ovary.
4	1	3,754	3	0.0	Ovaries taken out several hours after death.
	2	6,473	17	0.26	
Total.....		89,545	592	0.75* 0.66†	Total does not include Guinea Pigs 3 and 4.
Total of Guinea Pigs 3 and 4 .....		14,156	48	0.33	

\* Average percentage of mitoses.

† Absolute percentage of mitoses.

TABLE V.

*Percentage of Mitoses in Mature Follicles.*

Guinea pig No.	Ovary.	No. of cells.	No. of mitoses.	Percentage of mitoses.
				<i>per cent</i>
6		15,640	5	0.03
8	1	3,418	8	0.23
	2	5,387	4	0.07
Total.....		24,445	17	0.11* 0.06†

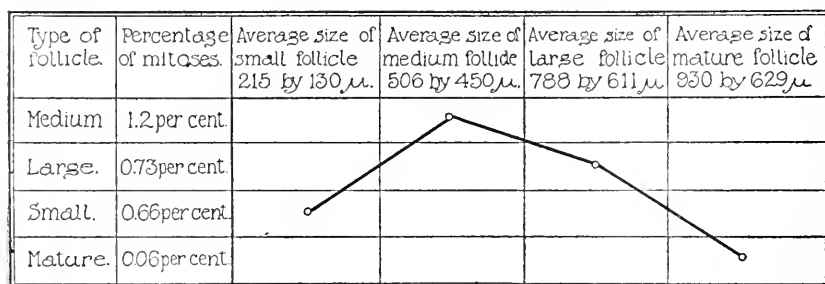
\* Average percentage of mitoses.

† Absolute percentage of mitoses.

cells and 17 mitoses were counted with an average percentage of mitoses for the follicles of the respective ovaries of 0.11, whereas the absolute number of mitoses per hundred cells is 0.06.

It follows, therefore, that mitoses are almost absent in mature follicles, which agrees with the observation of Loeb<sup>1</sup> and others that mitoses are very infrequent in mature follicles.

*Growth Curve.*—If we represent the variation in the growth energy in accordance with the development of the different follicles, we obtain a curve such as is represented in Text-fig. 1. We see that the growth energy increases with the development of the follicle from small to medium size, and here it reaches its maximum, then it again



TEXT-FIG. 1. Curve indicating the proliferative energy of follicles as a function of their size.

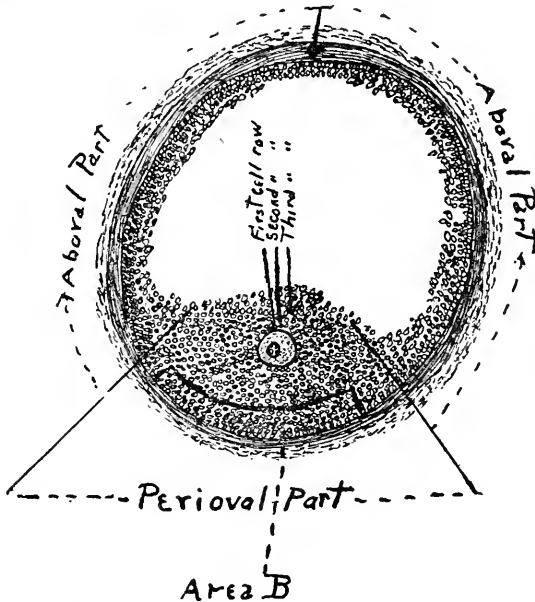
decreases, the growth energy of large follicles being at approximately the same level as that of the small follicles. A steep fall to mature follicles then takes place.

This curve bears some resemblance to that described by Robertson<sup>2</sup> and Ostwald<sup>3</sup> and some others for various growth processes. However, Robertson and Ostwald based their results not on percentage increases but on absolute increases. If the curves of Robertson and Ostwald were expressed in percentage increases they would differ markedly from ours. As Loeb has pointed out, it is probable that the decrease in growth energy of mature follicles is associated with greater cell differentiation in these follicles.

<sup>2</sup> Robertson, T. B., *Arch. Entwicklungsmechn. Organ.*, 1908, xxv, 581.

<sup>3</sup> Ostwald, W., in Roux, W., *Vorträge über Entwicklungsmechanik der Organismen*, Heft 5, Leipsic, 1908.

*Quantitative Determination of the Location of Mitoses in the Granulosa Cells of Follicles of Various Sizes.*—It has been observed by Loeb that the mitoses occurring in mature follicles are found in the discus proligerus. This observation suggested the possibility that the ovum stimulated the proliferation of the granulosa cells and made it desirable to investigate more closely the localization of mitoses in various kinds of follicles.



TEXT-FIG. 2. Areas in the ovarian follicle.

A number of follicles in the various ovaries having only a few granulosa cells surrounding the egg were selected. The number of mitoses occurring in the various cell rows around the egg was determined and, in addition, whenever a cavity had been formed the part of the granulosa in which the mitoses occurred was noted. Moreover, measured areas at various distances from the egg were compared as to the frequency of their mitoses.

In order to facilitate the description of our findings we found it necessary to divide the granulosa into several parts (Text-fig. 2). We divide the granulosa into two main parts: the perioval part which comprises the discus proligerus and the few cells distal to it which

are indicated on the diagram; the remaining part of the granulosa we call the aboval part. We also distinguish within the perioval part an area designated B on the diagram, of which the length is variable, but never exceeds the limitations of the perioval part, and the short diameter extends from the theca interna behind the egg towards it to the point where the cells begin to be more loosely arranged. We determined the relative frequency of mitoses in the peri- and aboval parts and in addition compared the number of mitoses per sq.  $\mu$  in area B with the aboval part.<sup>4</sup>

26 small follicles in which the egg was surrounded by from five to ten rows of granulosa cells, were analyzed in the manner just described, with the following result. 8 of the 26 follicles (30 per cent) have 100 per cent of their total mitoses occurring in the first cell row. 17, including the above 8 (65 per cent), have 50 per cent or more of their total mitoses occurring in the first cell row. 4 (15 per cent) have less than 50 per cent of their total mitoses occurring in the first cell row, but more mitoses near the egg than near the theca interna. 3 (11 per cent) have their greatest number of mitoses midway between the egg and the theca. 2 (7 per cent) have more of their total mitoses near the theca than near the egg.

It has been shown here that 65 per cent of the total mitoses occur in the first two cell rows, 30 per cent of which are in the first cell row. By total mitoses we understand all the mitoses observed by study of the follicle in serial section. By first cell row we mean the row of cells encircling the egg (Text-fig. 2).

Four small follicles, in which the space lined by the theca interna had an average measurement of 253 by 170  $\mu$  and in which there was early cavity formation, were studied. The findings were as follows:

1st cell row contains	16 per cent of the total mitoses of the follicles.
2nd " " "	5 " " " " " " " " " "
3rd " " "	10 " " " " " " " " " "
4th " " "	8 " " " " " " " " " "
5th " " "	6 " " " " " " " " " "
6th " " "	6 " " " " " " " " " "
7th " " "	0.8 " " " " " " " " " "
Remaining part of granulosa contains	44 " " " " " " " " " "

<sup>4</sup> Because of the variability of length of area B sometimes a few cells of the perioval part were included in the aboval part.

It is seen here that as the follicle increases in size the percentage of the total mitoses which occur in the first and second cell rows, decreases, yet 30 per cent of the mitoses still occur in the first three cell rows. The proliferative power of the cells near the theca is greater than in follicles of smaller size.

Next the results obtained in the study of nine small follicles, the cavities of which are of somewhat larger size than the preceding two kinds, are given. The average diameter of the space lined by the theca interna in these follicles was 215 by 132  $\mu$ ; the average size of the cavity 72 by 71  $\mu$ .

1st cell row contains	14	per cent of the total mitoses of the follicles.
2nd " " "	9	" " " " " " " " "
3rd " " "	8	" " " " " " " " "
4th " " "	1	" " " " " " " " "
5th " " "	9	" " " " " " " " "
6th " " "	3	" " " " " " " " "
9th " " "	1	" " " " " " " " "
Remaining part of granulosa contains	52	" " " " " " " " "

The results here are similar to those obtained in the preceding follicles. As the follicles increase in size there has been a gradual increase in the number of mitoses not in the immediate neighborhood of the egg.

The results of the analysis of the localization of mitoses in thirteen medium follicles, in which the average diameters of the space lined by the theca interna were 506 by 450  $\mu$ , are given below.

1st cell row contains	4	per cent of the total mitoses of the follicles.
2nd " " "	1	" " " " " " " " "
3rd " " "	3	" " " " " " " " "
4th " " "	2	" " " " " " " " "
5th " " "	4	" " " " " " " " "
6th " " "	2	" " " " " " " " "
7th " " " less than	1	" " " " " " " " "
Remaining part of granulosa contains	80	" " " " " " " " "

In medium follicles the remaining part of the granulosa includes a considerable number of cells in the perioval part of the follicle,

while in small follicles with a cavity the remaining part of the granulosa is made up almost entirely of cells in the aboval part.

In medium follicles the migration of the proliferating area from the first few cell rows encircling the egg to the cells near the theca behind the egg (see area B in Text-fig. 2), has been completed and now the greatest percentage of the mitoses of the follicle occurs in area B, as we shall see more specifically later on, and hence the majority of the 80 per cent of the mitoses in the remaining part of the granulosa occurs in this area.

The migration of the area of greatest mitotic activity reaches its maximum distance from the egg synchronously with the maximum growth energy of the follicle. Perhaps this is partly accounted for by the fact that the cells are farther apart and their number may be relatively smaller.

The analysis of the localization of mitoses in large follicles brought out the fact that the observation of Loeb that mitoses in mature follicles are limited to the discus proligerus, also to a certain extent holds good in large follicles which are not mature, as is shown by the following data.

Several large follicles were studied in the usual way and it was found that 43 per cent of their total mitoses occurred in the first two cell rows encircling the egg; 50 per cent occurred in the perioval (Text-fig. 2), and 7 per cent in the aboval part. The percentage of the total mitoses has increased in the first two cell rows coincidentally with the decrease in growth energy of the follicle.

The difference in the growth energy of the perioval and aboval parts of large follicles is much greater than in medium sized ones, as in the case of the former the proliferative power of the aboval part has come almost to a standstill. This agrees with the observation of Loeb that degenerative processes usually attack the cells of the discus proligerus last.

It seems reasonable to assume on the basis of the preceding data that the egg may furnish a direct stimulus to the granulosa cells, especially in view of the fact, as will be brought out later, that the site of greatest mitotic activity is always near the egg.

We next compared the frequency of mitoses in the peri- and aboval parts of the follicles. We also compared the number of mitoses in the

aboval part reduced to the sq.  $\mu$  equivalent of area B, with the latter, with the following results.

*20 Small Follicles.*

Perioval part contains 72 per cent of the total mitoses.

Aboval " " 26 " " " " " "

Area B contains 80 per cent of the total mitoses.

Aboval part, reduced to sq.  $\mu$  equivalent of area B,  
contains 19 per cent of the total mitoses.

*6 Medium Follicles.*

Perioval part contains 66 per cent of the total mitoses.

Aboval " " 33 " " " " " "

Area B contains 71 per cent of the total mitoses.

Aboval part, reduced to sq.  $\mu$  equivalent of area B,  
contains 25 per cent of the total mitoses.

*2 Large Follicles.*

Perioval part contains 93 per cent of the total mitoses.

Aboval " " 7 " " " " " "

Area B contains 85 per cent of the total mitoses.

Aboval part, reduced to sq.  $\mu$  equivalent of area B,  
contains 14 per cent of the total mitoses.

To compare area B with the aboval area, we determined the number of sq.  $\mu$  in each and multiplied the number of mitoses counted in area B by the number of times the aboval part exceeded area B in sq.  $\mu$ .

As a result of our study of the localization of mitoses in the granulosa cells we draw the following conclusions: (1) In all follicles the large majority of mitoses occurs in the perioval part. (2) In medium follicles where the percentage of mitoses is relatively highest, there are more mitoses in area B and in the aboval part than in large or small follicles. Moreover, in medium follicles there is a relative preponderance of cells some distance from the egg as compared with large and small, where they are more directly around the egg, showing that with increase in the percentage of mitoses in medium follicles, the granulosa cells not directly around the egg, gain relatively more in proliferative power than the cells around the egg.

*Growth of the Follicle under Pathological Conditions.*

We analyzed the proliferative power of follicles under two pathological conditions: (a) In follicles in which part of the granulosa cells had undergone degeneration by karyorrhexis, a condition which corresponds to early follicular degeneration; (b) secondly we investigated the condition in the follicles of hypotypical ovaries.

(a) *Growth Energy of Follicles in Beginning of Degeneration of Granulosa Cells.*—Only follicles which showed rather extensive degeneration by karyorrhexis were considered. In this form of atresia the degeneration is usually most evident in the cell rows nearest the cavity of the follicles. The growth energy in these follicles was determined in the same way as in normal ones and only those cells were counted in which there was no visible degeneration. The follicles and their cavities were usually somewhat larger than medium follicles. The results of the examination of twenty-seven follicles with partial granulosa degeneration are given in Table VI.

TABLE VI.  
*Percentage of Mitoses in Degenerating Follicles.*

Guinea pig No.	Ovary.	No. of cells.	No. of mitoses.	Percentage of mitoses.	Remarks.
				<i>per cent</i>	
5	1	6,789	14	0.20	Corpora lutea burned out. Found dead.
	2	8,310	45	0.54	
6	1	7,247	10	0.13	
	2	2,304	0	0.0	
2	1	6,450	29	0.44	
	2	7,143	50	0.69	
3		2,604	0	0.0	
4	1	4,870	0	0.0	
	2	4,036	1	0.02	
Total.....		49,753	149	0.22* 0.29†	

\* Average percentage of mitoses.

† Absolute percentage of mitoses.

It is shown here that both the absolute and average percentage of mitoses of the cells in these follicles which are not yet degenerating are much lower than in normal follicles of the same size.



It was observed during this study that the majority of the mitoses occurs in the cells of the discus proligerus and that the percentage of mitoses is proportionate to the amount of degeneration; the more extensive the degeneration the weaker is the proliferative power of the cells which have not yet degenerated.

(b) *Growth Energy of Follicles of Hypotypical Ovaries.*—Under this designation Loeb<sup>5</sup> has described ovaries which he has observed under several conditions, for instance, after the burning out of corpora lutea, etc. Hypotypical ovaries are smaller than normal; they are characterized by an early connective tissue atresia of follicles, taking place while the follicles are quite small, and hence there are no large, no mature follicles, and for the time being the ovary is sterile. It was our intention to determine if possible whether the failure of follicles in such ovaries to grow past small size was due to a diminished proliferative power of the granulosa or to an increased cell destruction. We may mention here that granulosa degeneration by karyorrhexis does not take place in the hypotypical ovaries except in isolated cells and not *en masse* as in normal follicles.

The results obtained in fifty-five follicles, in six hypotypical ovaries of three animals, are presented in Table VII. As has already been mentioned, the follicles in hypotypical ovaries become atretic at so early a stage of their development that only small follicles are seen well preserved. The average diameters of the space lined by the theca interna, of the follicles whose figures appear in Table VII, are as follows.

Long diameter of space lined by theca interna from	277	to	131 $\mu$ ;	average	209 $\mu$ .
Short " " " " " " " "	246	"	211 $\mu$ ;	"	178 $\mu$ .
Long " " cavity from	188	to	35 $\mu$ ;	average	91 $\mu$ .
Short " " " " " " " "	208	"	54 $\mu$ ;	"	135 $\mu$ .

Table VII shows that the average percentage of mitoses (0.57) and the absolute percentage (0.55) in the hypotypical follicles are nearly the same, but slightly lower than in normal follicles of the same size.

In the granulosa cells of the hypotypical follicles, those cells which remain preserved show almost the same proliferative power as the

<sup>5</sup> Loeb, *Zentr. Physiol.*, 1911-12, xxv, 342.

TABLE VII.

*Percentage of Mitoses in Follicles of Hypotypical Ovaries.*

Guinea pig No.	Ovary.	No. of cells.	No. of mitoses.	Percentage of mitoses.
				<i>per cent</i>
9	1	5,661	12	0.21
	2	5,668	24	0.42
10	1	9,491	51	0.53
	2	2,463	27	1.0
11	1	10,099	57	0.56
	2	8,703	61	0.70
Total.....		42,085	232	0.57* 0.55†

\* Average percentage of mitoses.

† Absolute percentage of mitoses.

granulosa cells of normal follicles. But it would be incorrect to state that the granulosa as such in hypotypical follicles shows the same proliferative power. Evidently a considerable number of cells which remain preserved in normal follicles undergo premature solution in hypotypical follicles and these cells no longer proliferate. Therefore the absolute number of mitoses in follicles of hypotypical ovaries is of course diminished. It is, however, of interest that the cells which remain show only slightly less proliferative power than the cells of normal follicles.

We also made comparative studies of normal and hypotypical follicles. (a) We compared the localization of mitoses in the two kinds of follicles and found that the localization phenomenon in hypotypical follicles parallels that of normal follicles of the same size. (b) We compared the size of the nuclei in normal and hypotypical follicles by measuring them at various phases in the granulosa in a number of follicles of each kind. It was found that on the whole the nuclei of normal follicles slightly exceeded in size the hypotypical. (c) The relative sizes of the cavities of normal and hypotypical follicles were compared and we found that the cavity of the hypotypical is relatively about twice as large as that of the normal follicle of the same size.

## SUMMARY AND CONCLUSIONS.

1. The growth energy of the granulosa cells in the ovarian follicles of normal guinea pig ovaries varies with the size of the follicle, and follows a definite growth curve. The growth energy in small follicles is relatively low, then a gradual rise takes place with increase in size of the follicle up to a maximum which is reached when the follicle attains medium size. This ascending part of the curve is followed by a gradual fall in growth energy until in large follicles the proliferative power has fallen almost to the level of small follicles. Synchronously with maturity of the follicles there is an abrupt fall in growth energy to near the zero point.
2. There is a distinct localization of mitoses in the follicle. The large majority of mitoses is found in the perioval part. With increase in the growth energy which characterizes medium follicles, there is an increase in the proliferative power of the cells more distal to the egg, but still near the egg. We may therefore conclude on the basis of our observations and on the previous observations of Loeb, that the egg exerts a stimulus, which causes proliferation of the granulosa cells.
3. Under pathological conditions certain changes occur in the growth energy of the follicle: (a) Granulosa degeneration causes in the living cells a diminution in the proliferative power which is proportionate to the extent of the degeneration. (b) Hypotypical follicles have approximately the same or only slightly diminished proliferative power as normal ones, and thus their failure to grow is essentially due to disintegration of the granulosa cells.



# THE RELATION OF APICAL TUBERCULOSIS OF ADULTS TO THE FOCAL TUBERCULOSIS OF CHILDREN.

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PLATES 20 AND 21.

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In a previous article<sup>1</sup> I have shown that the lungs of almost every individual who has reached adult life (18 years) contain foci of tuberculosis which, occurring in any part of the lung and associated with tuberculosis of the regional lymphatic nodes, have the characters of pulmonary tuberculosis in childhood. In infancy tuberculosis is almost invariably fatal, but between the ages of 2 and 18 years tuberculous lesions of the lung heal with increasing frequency as age advances. Almost every child is spontaneously inoculated or "vaccinated" with tuberculosis. The early experiments of Koch<sup>2</sup> confirmed by subsequent observers have shown that a first infection with tuberculosis modifies the course of a second infection. Koch's fundamental experiment showed that the injection of tubercle bacilli into the subcutaneous tissue of animals already infected with tuberculosis caused a reaction at the site of infection more severe than that of the first infection, but the lesion did not progress and there was no involvement of the regional lymphatic nodes. Experience acquired by attempted immunization of small animals and by the inoculation of cattle with tubercle bacilli of human type has demonstrated that immunity conferred by a first infection with tubercle bacilli is relative and not absolute, and tends to diminish and perhaps disappear after complete healing of the tuberculous lesions caused by inoculation.

Fatal pulmonary tuberculosis of adults and of older children with few exceptions has its origin in the apices of the lungs and like the second infection of animals, produced by Koch and others, is unaccompanied by caseous lesions of the regional lymphatic nodes. These characters serve to separate adult from infantile tuberculosis. The incidence of focal and apical tuberculosis of the lungs in a series of autopsies on children and adults has already been shown.<sup>3</sup> It

<sup>1</sup> Opie, E. L., *J. Exp. Med.*, 1917, xxv, 855.

<sup>2</sup> Koch, *Deutsch. med. Woch.*, 1891, xvii, 101.

<sup>3</sup> See Tables I and III of the previous article,<sup>1</sup> pp. 857 and 865.

is probable that the percentage of focal infections in adults is greater than that indicated by the tables<sup>3</sup> and closely approximates 100 per cent, for in those instances in which no pulmonary lesion was found tuberculosis of lymphatic nodes in two instances demonstrated only by microscopic examination suggested the probability that corresponding pulmonary lesions had been overlooked. Even if this view is incorrect it may be assumed that tuberculosis of lymphatic nodes or of other organs would exert an influence upon resistance similar to that of primary pulmonary tuberculosis. Nevertheless the identification of a focal pulmonary lesion accompanied by tuberculosis of regional lymphatic nodes in the lungs of an adult is significant, for, as the tables show, it indicates that tuberculosis has been acquired in childhood and presumably antedates an apical lesion with which it may be associated. Orth<sup>4</sup> states that the experience of pathological anatomy contradicts the view that there is in every instance of phthisis an older lesion which has had its origin in childhood, and in most individuals who have died of phthisis he has found no such older lesion.

In view of the character of the material which has been available for study it has been the primary purpose of the present investigation to study the relation of non-lethal apical tuberculosis to the focal tuberculosis of childhood. Incidentally there has been an opportunity to compare healed or encapsulated apical tuberculosis with progressive apical phthisis and to determine as far as possible whether apical tuberculosis has a greater tendency to a fatal issue in those who have escaped infection in childhood.

Apical lesions have been regarded as tuberculous when microscopic examination has demonstrated the presence of recognizable tubercles, when caseation has occurred, or when calcified nodules which represent the site of former caseation are embedded within fibrous tissue. There is little reason for doubting that the fibrous scars which often pucker the apical pleura and usually extend as wedge-shaped masses into the lung substance have their origin in tuberculous infection, but in the absence of recognizable tubercles, caseation, or calcification these lesions have not been designated tuberculous. A healed tuberculous focus consisting of fibrous tissue in which are

<sup>4</sup> Orth, J.. *Drei Vorträge über Tuberkulose*, Berlin, 1913.

calcified nodules may be found at one apex, whereas at the other there is only fibrous induration. There is more doubt concerning the nature of fibrous induration which not infrequently involves the pleura over a circumscribed area at the apex of the lung and does not penetrate into the underlying lung tissue. This pleural induration is not infrequently found at the apex of one lung, whereas there is a fibrous scar at the apex of the other lung, and occasionally with a caseous lesion of one lung the opposite apical pleura is indurated.

Table I shows the frequency with which apical tuberculous lesions have occurred at various ages in autopsies on adults and children.

TABLE I.

Age.	No. of autopsies.	Active apical tuberculosis.	Encapsulated apical tuberculosis.	Total apical pulmonary tuberculosis.		
Children.						
yrs.			per cent	per cent	per cent	
Under 1	43	—	—	—	—	
1- 2	16	—	—	—	—	
2- 5	14	—	—	—	—	
5-10	11	—	—	—	—	
10-18	9	1	11.1	—	11.1	
Adults.						
18-30	6	1	16.7	—	—	16.7
30-50	23	1	4.3	2	8.7	13.0
50-70	15	1	6.7	3	20.0	26.7
70+	6	—	—	3	50.0	50.0

The number of cases in each group is small and the percentages represent only crudely the incidence of the various lesions at various ages. Nevertheless they show that the incidence of encapsulated and healed tuberculosis increases with increasing age whereas there is no similar increase in the incidence of active tuberculosis.

Table II shows the frequency of induration affecting the apical pleura and of fibrous scars at the apices of the lungs.

The table shows that these purely fibrous lesions like healing or healed apical lesions which are demonstrably tuberculous are uncom-

TABLE II.

Age.	No. of autopsies.	Induration of apical pleura.	Scars within apex.		
Children.					
<i>yrs.</i>			<i>per cent</i>		<i>per cent</i>
Under 1	43	—	—	—	—
1- 2	16	—	—	—	—
2- 5	14	—	—	—	—
5-10	11	—	—	—	—
10-18	9	—	—	—	—
Adults.					
18-30	6	1	16.7	1	16.7
30-50	23	4	17.4	4	17.4
50-70	15	6	40.0	5	33.3
70+	6	2	33.3	1	16.7

mon in early adult life, but increase in frequency as age progresses. They do not increase after the age of 70 years because at this time pulmonary apices of over half of all individuals are occupied by frankly tuberculous lesions.

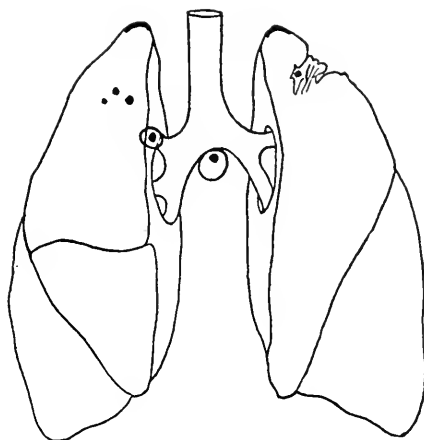
Apical pleural induration in all instances has been accompanied by demonstrably tuberculous lesions in some other part of the lungs.

Table I contains eight instances of encapsulated apical tuberculosis. In these lungs focal tuberculous lesions have been found in association with the apical lesions and without doubt have antedated them. The situation of the focal lesion in relation to the apical lesion is shown by Text-figs. 1 to 7<sup>5</sup>, <sup>6</sup> and in x-ray plates (Figs. 1, 2, and 3). These focal lesions have been in most instances firmly calcified and completely healed; in five (Text-figs. 1 to 3<sup>5</sup>, <sup>6</sup>) of eight instances the focal nodules have been of almost stony hardness. In two instances (Text-figs. 4 and 5) the focal lesions have been caseous and partly calcified (mortar-like), and in one instance (Text-fig. 6) calcified, partially calcified, and encapsulated caseous foci have been found in the lungs.

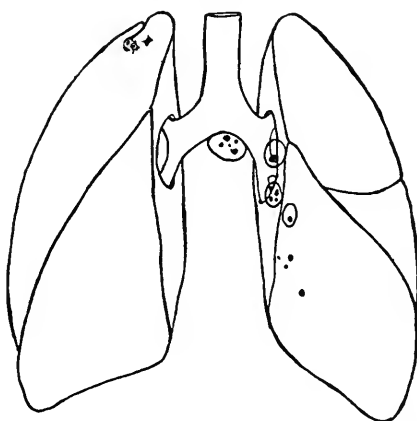
<sup>5</sup> See also Text-fig. 7 of the previous article.<sup>1</sup>

<sup>6</sup> See also Text-fig. 8 of the previous article.<sup>1</sup>

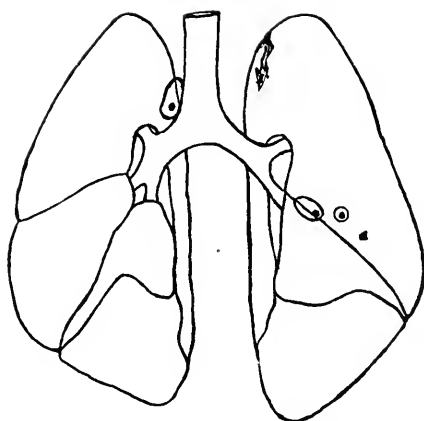




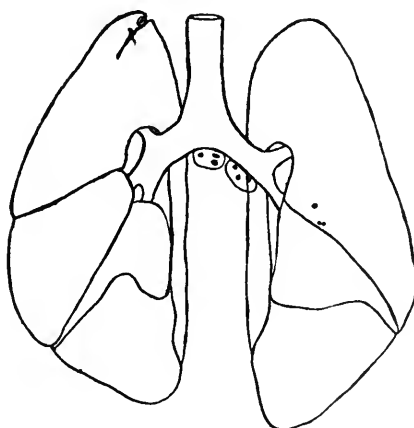
TEXT-FIG. 1. Anterior view of the lungs of a white woman, age 54 years, who died after hysterectomy for carcinoma of the cervix. There are three encapsulated nodules in the upper part of the right upper lobe and calcified nodules are found in lymph glands at the hilus of the right upper lobe and at the bifurcation of the trachea. There is an indurated fibrous area containing a caseous focus situated in contact with the pleura of the external surface of the left upper lobe a short distance below the apex of the lung. The pleura over this area is puckered and at one place drawn far into the substance of the lung.



TEXT-FIG. 2. Posterior view of the lungs of a colored woman, age 60 years, who died of chronic endocarditis, general arteriosclerosis, and cerebral embolism. In the right lower lobe below the pleura of the external surface are three small encapsulated calcified nodules and in the substance of the lobe is a fourth calcified nodule. In the lymph nodes at the hilus of the right lower lobe and at the bifurcation of the trachea are calcified nodules. Just below the apex of the left upper lobe continuous with the indrawn pleura is a thick strand of pigmented fibrous tissue which passes into the substance of the lobe and is continuous with an area of fibrous induration containing two caseous areas.



TEXT-FIG. 3. Median view of the lungs of a white man, age 61 years, who died of hypernephroma of the kidney with metastases to the liver, lymph nodes, and bone, general arteriosclerosis, chronic passive congestion of viscera, ascites, and pleural effusion. There is a calcified nodule in the lower part of the upper left lobe and calcified nodules occur in adjacent lymphatic nodes and in a lymphatic node above the right bronchus. At the apex of the left lung continuous with the pleura of the median surface just below the apex is a narrow mass of fibrous tissue containing a small caseous area.



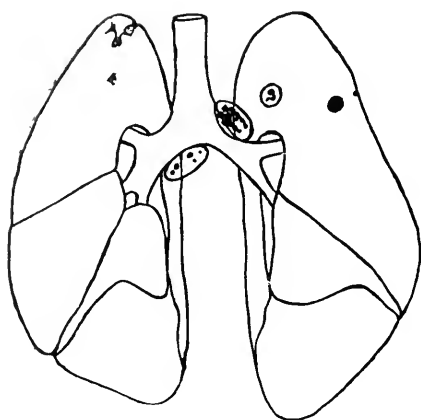
TEXT-FIG. 4. Median view of the lungs of a white man, age 73 years, who died with carcinoma of the sigmoid flexure of the colon, intestinal obstruction, perforation of the intestine and peritonitis, arteriosclerosis, chronic nephritis with granular kidneys, hypertrophy of the heart, and myocarditis. In the lower part of the left upper lobe are three encapsulated partially calcified caseous nodules. In lymph nodes below the left bronchus and at the bifurcation of the trachea are partially calcified caseous nodules. Just below the apex of the right upper lobe extending from the indrawn pleura into the substance of the lung is a coarse strand of fibrous tissue within which is a partially calcified caseous nodule.

The apical lesions have in several instances occupied the greater part of one or both apices and have consisted of numerous caseous tubercles embedded in fibrous tissue pigmented black by inhalation pigment (Figs. 1 and 3 and Text-figs. 6 and 7<sup>5, 6</sup>). In other instances the lesion has consisted of strands or masses of fibrous tissue in which are caseous or caseous and partially calcified nodules (Fig. 2 and Text-figs. 1 to 5). In every instance the lesion is at some point in contact with the pleural surface near the apex of the lung. The pleura at the site of contact with the lesion is puckered and not infrequently deeply drawn into the lesion (Text-figs. 1, 2, and 4).

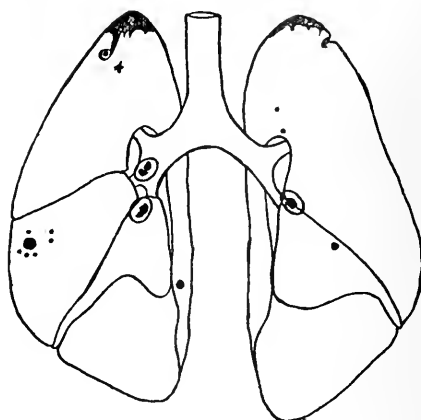
In the instances which have been cited slowly progressive tuberculosis characterized by abundant formation of fibrous tissue has occurred at the apex of the lung and has been associated with focal pulmonary lesions doubtless acquired in childhood. In one instance slowly progressive tuberculosis of organs other than the lung, namely the adrenals, accompanied focal encapsulated caseous lesions of the lower part of the upper lobe of the right lung and encapsulated caseous lesions of the regional lymphatic nodes. Pigmentation of the skin with general weakness had been noted 4 years before death and at autopsy the adrenals were found transformed into masses of dense fibrous tissue containing caseous spots. It is noteworthy that the lesions in this instance were all caseous and encapsulated; beginning calcification of the pulmonary lesion indicated that it was older than those found in other organs.

In one instance (Text-fig. 7) chronic apical tuberculosis in a woman aged 39 years was active and implicated a considerable part of the left lung but found in association with carcinoma of the stomach with metastases in the ovaries, liver, and peritoneum was not the cause of death. In the upper and lower lobes on the right side there were firmly calcified nodules and the regional lymphatic nodes contained similar foci. The presence of abundant pigmented fibrous tissue was index of the slow progress of the lesion. It is not improbable that associated cancer produced conditions favorable to the extension of an apical lesion.

One instance of fatal tuberculosis of the lung complicated by the presence of chronic peritonitis and chronic unilateral pleurisy does not admit of any conclusion concerning the primary seat of infection;



TEXT-FIG. 5.



TEXT-FIG. 6.

TEXT-FIG. 5. Median view of the lungs of a white man, age 40 years, who died of anthracosis of the lungs, hypertrophy of the right heart, and central necrosis of the liver. In the left upper lobe are two caseous partly calcified nodules, the larger 1.2 cm. across; in regional lymphatic nodes within the lung above the right bronchus and at the bifurcation of the trachea are caseous partly calcified nodules. At the apex of the right lung continuous with the puckered pleura is deeply pigmented fibrous tissue containing three small calcified nodules and one caseous area. In the substance of the upper right lobe near the apex is a fibrous area in which are two calcified nodules.

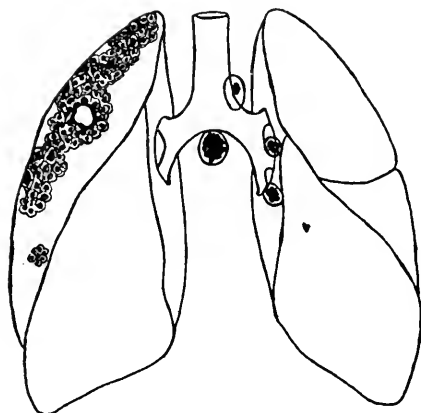
TEXT-FIG. 6. Median view of the lungs of a white man, age 74 years, who died after resection of the cecum for carcinoma with purulent peritonitis, general arteriosclerosis, and chronic nephritis. In the right middle lobe is a calcified nodule 1.2 cm. across, and nearby just below the pleura are a number of small calcified nodules. In the right lower lobe is a partially calcified encapsulated caseous nodule. At the hilus of the right middle and lower lobes are two lymphatic nodes containing encapsulated caseous foci. In the left lower lobe is an encapsulated partially calcified caseous focus, and a similar focus occurs in an adjacent lymph node. Two small calcified nodules occur below the pleura of the median surface of the left upper lobe. The apex of the right upper lobe is occupied by a consolidated fibrous area 1.5 cm. in thickness. It contains several small smooth-walled cavities about 3 mm. in diameter. A fibrous strand extends downward from this indurated area and surrounds a caseous focus. A second caseous focus surrounded by fibrous tissue occurs in the lung substance nearby. The apex of the left upper lobe shows fibrous induration similar to that on the right side forming a zone 0.5 cm. in thickness.

there was a small caseous focal lesion in the upper lobe of the right lung but the lymphatic nodes at the hilus of the lobe contained no tuberculous lesions. This focal lesion was recognized by its shadow 2 mm. across cast upon an x-ray plate and doubtless contained some calcium salts, although there was no macroscopic evidence of calcification. With chronic tuberculous pleurisy there was effusion into the left pleural cavity, atelectasis of the left lung, and chronic tuberculous peritonitis with adhesions firmly matting together the intestines. There was active caseous tuberculosis of the right lung most advanced near the median surface of the organ where the lung had been subjected to the pressure of the effusion in the left pleural cavity. There was some fibrous induration at the apex of the compressed left lung. The tuberculous lesion of the peritoneum and pleura was characterized by the new formation of abundant fibrous tissue and had pursued a chronic course.

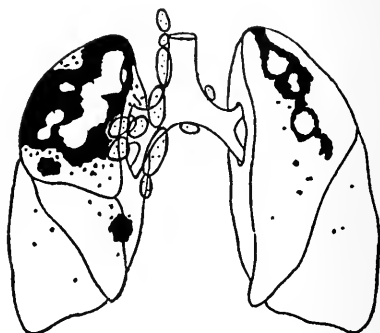
It is noteworthy that in two instances pulmonary tuberculosis has been the cause of death and in neither of these cases were pre-existing focal lesions found in the lungs.

Text-fig. 8 is a diagram of tuberculous lesions in the lungs of a child 11 years of age. Almost the entire right upper lobe including the apex is consolidated, in large part caseous, and contains numerous cavities. The upper part of the left upper lobe contains similar consolidated areas in which are cavities. The remainder of the lungs contains scattered tubercles. Lymphatic nodes at the hilus of the right lung and along the right side of the trachea are much enlarged and succulent; there is no evident caseation, but microscopic examination shows the presence of scattered caseous tubercles. The lungs were cut into thin sections and no focal lesions were recognized. There were tuberculosis of the larynx, tuberculous ulcers of the intestine, and tuberculous peritonitis. The von Pirquet tuberculin test was negative 6 weeks before death.

The second instance of fatal tuberculosis (Text-fig. 9) is a remarkable example of acute rapidly progressive disease terminating with tuberculosis of the bodies of the lumbar vertebrae, psoas abscess, tuberculosis of retroperitoneal lymphatic nodes, tuberculosis of the thoracic duct, and disseminated miliary tuberculosis in a man with no old focal tuberculous lesion of the lung. An apical lesion which



TEXT-FIG. 7.



TEXT-FIG. 8.

TEXT-FIG. 7. Posterior view of the lungs of a white woman, age 39 years, who died of carcinoma of the stomach and metastatic carcinoma of the ovaries, liver, and peritoneum. There is chronic pulmonary tuberculosis with abundant formation of fibrous tissue at the upper part of the left upper lobe; cavities have been formed. Several scattered areas of similar consolidation occur in other parts of the lungs; a consolidated partially caseous area in the mid-part of the right lung has been omitted from the diagram. There is an encapsulated calcified nodule 3.5 cm. across in the upper part of the right lower lobe; calcified foci are found in lymphatic nodes at the hilus of the same lobe, at the bifurcation of the trachea, and above the right bronchus.

TEXT-FIG. 8. Anterior view of the lungs of a white child, age 11 years, who died of pulmonary tuberculosis with cavity formation, tuberculous ulceration of the larynx, tubercles in tonsil, intestine, and liver, and tuberculous peritonitis. The von Pirquet tuberculin reaction was negative 6 weeks before death. There is tuberculosis with cavity formation involving both apices but much more advanced on the right side. Lymphatic nodes at the hilus of the right upper lobe, about the right bronchus, and along the right side of the trachea are much enlarged and contain tubercles.

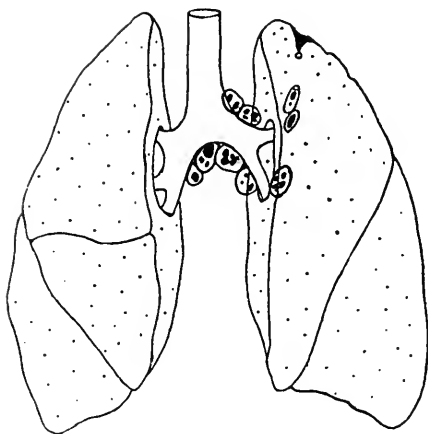
had undergone caseation was found in the left lung immediately below the pleura. In continuity with this lesion was a small dilated bronchus, of which the wall was caseous. Below the level of this caseous bronchus were numerous patches of tuberculous bronchopneumonia. The remainder of both lungs was occupied by numerous miliary tubercles. An x-ray plate (Fig. 4) demonstrates the absence of calcified nodules within the substance of the lung or in the regional lymphatic nodes.

It is particularly significant that in the absence of focal pulmonary lesions lymphatic nodes within the substance of the left upper lobe in proximity to the apical lesion and at the hilus of the lobe are enlarged and caseous (Text-fig. 9). It may be assumed that in the absence of preexisting tuberculosis the apical lesion has caused active tuberculosis of the regional lymphatic nodes. The apical lesion has acted as a first infection.

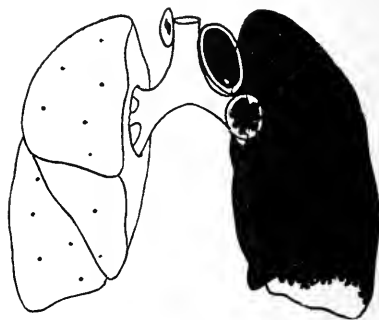
This relation of the regional lymphatic nodes to a first infection with tuberculosis is well illustrated by the spontaneous occurrence of tuberculosis in a macaque monkey kept in confinement. The entire left lung with the exception of a small area at the base is the site of tuberculous pneumonia with widespread caseation (Text-fig. 10). The lymphatic nodes at the hilus of the affected lung are greatly enlarged and partially caseous.

The relation of the focal tuberculous lesions usually acquired in childhood and of the corresponding lesions of regional lymphatic nodes to coexisting apical tuberculosis furnishes no evidence that the apical lesion is derived from the preexisting focal lesion. In most instances the focal pulmonary lesion and the accompanying lesions of lymphatic nodes have been firmly calcified and completely healed whereas the apical lesion is caseous (Text-figs. 1 to 3<sup>5, 6</sup>).

There is little probability that tuberculous infection has been transmitted by way of the lymphatics from the focal lesion of the lung or of the regional lymphatic nodes to the apex. The large lymphatic trunks of the lung fall into two groups; namely, superficial collecting trunks which, situated below the pleura, pass to the lymphatic nodes at the hilus of the lung, and deep collecting trunks which follow the bronchi, the pulmonary arteries, and the pulmonary veins to the nodes at the hilus of the lungs. It is not improbable that



TEXT-FIG. 9.



TEXT-FIG. 10.

TEXT-FIG. 9. Anterior view of the lungs of a colored man, age 28 years, who died of pulmonary tuberculosis, tuberculosis of the body of the second lumbar vertebra, psoas abscess, tuberculosis of retroperitoneal lymphatic nodes, tuberculosis of the thoracic duct, and disseminated miliary tuberculosis. The lungs are studded with miliary tubercles. At the apex of the left lung below the pleura of the external aspect of the lung is a caseous area 2 by 2 cm. on the surface extending in wedge-shaped form 1.5 cm. into the lung. At the apex of the wedge is a dilated bronchus with caseous wall. In the substance of the left upper lobe in a line between the tuberculous lesion in the apex and the hilus of the lung are enlarged partially caseous lymphatic nodes. About the left bronchus and at the bifurcation of the trachea are similar freshly caseous enlarged lymphatic nodes.

TEXT-FIG. 10. Anterior view of the lungs of a macaque monkey which died of tuberculous pneumonia involving almost the entire left lung. Lymphatic nodes at the hilus of the left lung are greatly enlarged and caseous.



lesions of lymphatic trunks or of lymphatic nodes may cause a reversal of the current within lymphatics. In four instances (Text-figs. 1, 2, and 7<sup>6</sup>) focal pulmonary lesions have been situated in the right lung whereas there has been an apical lesion only on the left side. In two instances (Text-fig. 4<sup>5</sup>) with focal lesions in the left lung apical lesions have been found only on the right side. The probability of transmission by lymphatic channels from the lung or lymphatic nodes on the one side to the apex of the opposite lung is scant.

#### SUMMARY.

The age incidence of focal tuberculous lesions of the lungs demonstrates that they have their origin in most instances in childhood. Focal lesions which heal have been found at all ages after the 2nd year of life, but in more than half of all individuals these lesions are acquired between the ages of 10 and 18 years. In the period between 18 and 30 years at least 85 per cent of all individuals have acquired focal tuberculous lesions. The occurrence of tuberculous infection in the lungs, in regional lymphatic nodes, or in some other organs of the body such as the gastrointestinal tract and its lymphatic system, is nearly universal but doubtless a few individuals escape. That focal tuberculous lesions of the lung are occasionally acquired during adult life is shown by the slight increase in the proportion of those with these lesions as age increases from 18 years to old age.<sup>3</sup>

Apical lesions of the lung make their appearance in later childhood and occur with increasing frequency from adolescence to old age (50 per cent). After the 2nd year of life focal tuberculous lesions occurring in situations other than the apices of the lungs tend to heal and after the 10th year focal lesions are almost invariably encapsulated and latent or healed. Fatal tuberculosis after the 10th year is with few exceptions apical in origin. The apices are not only more susceptible to infection in later life but once infected afford less resistance to the extension of the lesion.

The present series of cases has furnished opportunity to observe the character of the apical lesion in lungs of individuals previously infected with tuberculosis. With one exception the apical lesion (in eight instances) has pursued a chronic course and, encapsulated

by fibrous tissue, has remained limited to the extreme apex of the lung. In one instance in a woman with advanced malignant disease chronic pulmonary tuberculosis has been progressive. Tuberculosis of the apices in those who have previously acquired a focal tuberculous lesion has pursued a chronic course and in most instances has remained latent or has completely healed.

A very small group of instances of fatal pulmonary tuberculosis suggests that apical lesions in those who have not undergone previous infection may assume an unusually severe character. One instance of apical tuberculosis unaccompanied by focal lesions and followed by tuberculosis of the thoracic duct and disseminated miliary tuberculosis has been especially significant. Apical tuberculosis unaccompanied by evidence of preexisting tuberculosis may be accompanied by tuberculosis of the regional lymphatic nodes, whereas apical tuberculosis in an individual with a preexistent focal tuberculous lesion is not followed by tuberculosis of adjacent lymphatic nodes. It is well known that tuberculosis in previously uninfected animals is followed by tuberculosis of adjacent lymphatic nodes, whereas a second infection fails to implicate the regional lymphatic nodes. This relation has been well illustrated by the lungs of a monkey which acquired in confinement acute tuberculous pneumonia limited to the left lung; the lymphatic nodes on this side were greatly enlarged and caseous.

The following observations indicate that apical tuberculosis of adults is not the result of infantile tuberculosis but is caused by subsequent infection: (a) Apical tuberculosis does not have its highest incidence, in accordance with common belief, in early adult life when focal infections acquired in childhood are relatively fresh and active but is more common in later life when the focal lesions of childhood have in most instances completely healed. It is noteworthy that most of these apical lesions of later life pursue a chronic course and are discovered at autopsy in individuals who have died from other causes. (b) The well characterized lesions of tuberculosis acquired in childhood and found in adults with apical lesions are almost invariably calcified and healed. The apical lesion is in most instances relatively fresh and caseous whereas the focal pulmonary lesion and associated lesions of regional lymphatic nodes exhibit no evidence

of activity. (c) In a large proportion of instances of associated focal and apical tuberculosis the focal lesion is in one lung, whereas the apical lesion is limited to the opposite apex. This relation affords no support to the view that tuberculous lesions may be transmitted to the apex by way of the lymphatics.

#### EXPLANATION OF PLATES.

##### PLATE 20.

FIG. 1. X-ray plate of the lungs of a man, age 74 years. There is a consolidated area at the left apex containing caseous foci impregnated with enough calcium salts to cast shadows upon the plate. In the right lower lobe and in adjacent lymphatic nodes are completely healed firmly calcified nodules.<sup>7</sup>

FIG. 2. X-ray plate of the lungs of a man, age 40 years. The positions of the right and left lungs are reversed in the plate. At the right apex continuous with the pleura are strands of fibrous tissue in which occur small caseous and calcified areas. In the right upper lobe below the apex is a small fibrous patch containing two caseous partially calcified spots. In the left upper lobe near the inner surface is a firmly calcified encapsulated nodule 1 cm. across. Caseous areas of mortar-like consistency occur in regional lymphatic nodes. No nodule was found, after prolonged search, corresponding to the shadow 2 cm. across in the lower right lobe. Compare with Text-fig. 5 from the same lungs.

##### PLATE 21.

FIG. 3. X-ray plate of the lungs of a man, age 74 years. The positions of the right and left lungs are reversed in the plate. At the apex of the right lung there is an indurated area containing a caseous spot. The apex of the left lung is indurated. In the right middle and lower lobes, in the left lower lobe, and in regional lymphatic nodes are calcified, partially calcified, or caseous nodules. Compare with Text-fig. 6 from the same lungs.

FIG. 4. X-ray plate of the lungs of a man, age 28 years, who died of acute miliary tuberculosis. There are no shadows suggesting the presence of calcified nodules in the lungs or in their lymphatic nodes. Compare with Text-fig. 9 from the same lungs.

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<sup>7</sup> Text-fig. 8 of the previous article<sup>1</sup> is a diagram of these lungs.



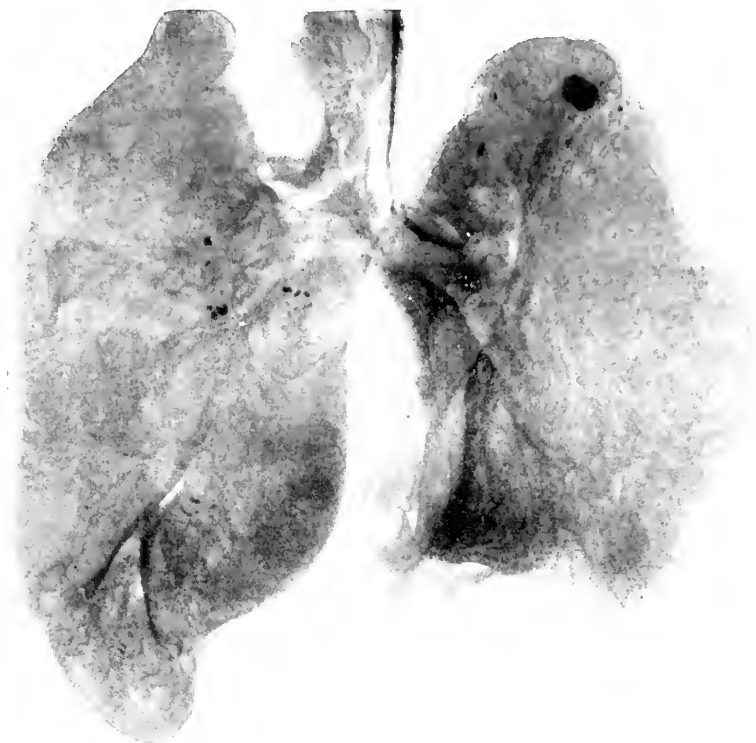


FIG. 1.



FIG. 2.

(Opie: Tuberculosis of adults and children.)



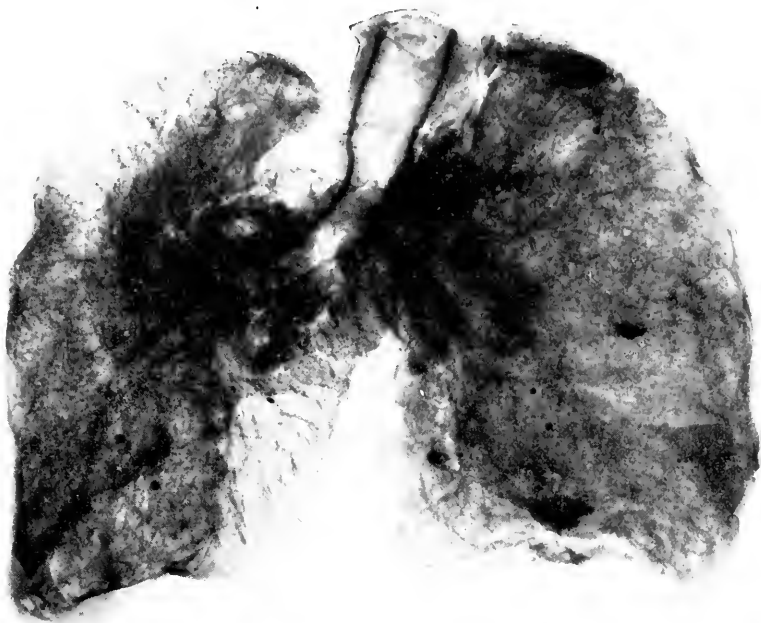


FIG. 3.

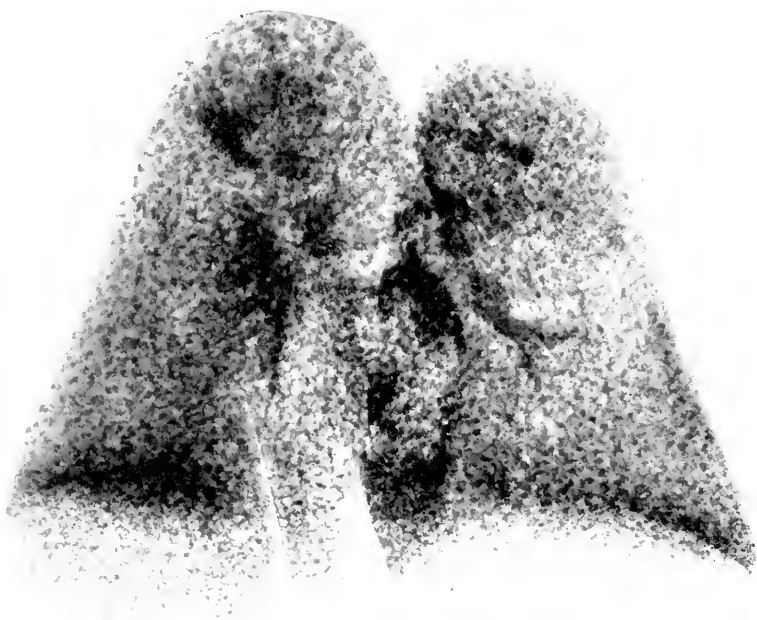


FIG. 4.

(Opie: Tuberculosis of adults and children.)





## CICATRIZATION OF WOUNDS.

### IX. INFLUENCE ON THE HEALING OF WOUNDS OF VARIATIONS IN THE OSMOTIC TENSION OF THE DRESSING.

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In previous articles it has been shown that the curve representing the process of cicatrization of an aseptic wound is geometric,<sup>1, 2</sup> and can be calculated by the formula of du Noüy. The comparison of the calculated and observed curves alters the study of the effect upon cicatrization of a substance applied to the surface of the wound. If the daily decrease of the area of a wound is known, and if the wound is maintained in a condition of surgical asepsis, the modifications in the rate of the healing process can be attributed to the special action of the substance applied, and the extent of this action can be accurately measured.

Up to the present it has not been known to what degree the rate of cicatrization of a wound can be affected by the dressing. Surgeons have studied the influence of so called healing substances when applied to wounds, but no precise conclusion has resulted from these observations. No method existed of measuring exactly the surface of a wound and of calculating with any degree of accuracy the rate of the healing process. On the other hand, the bacteriological condition of the wounds experimented upon was never taken into consideration. Such modifications of the rate of healing as were noticed might be equally attributable to the action of the substance employed upon the bacteria of the wound as to the tissues themselves.

<sup>1</sup> Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

<sup>2</sup> du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451.

The following experiments were undertaken to ascertain whether modifications in the osmotic tension of the dressing exert an influence on the rate of repair.

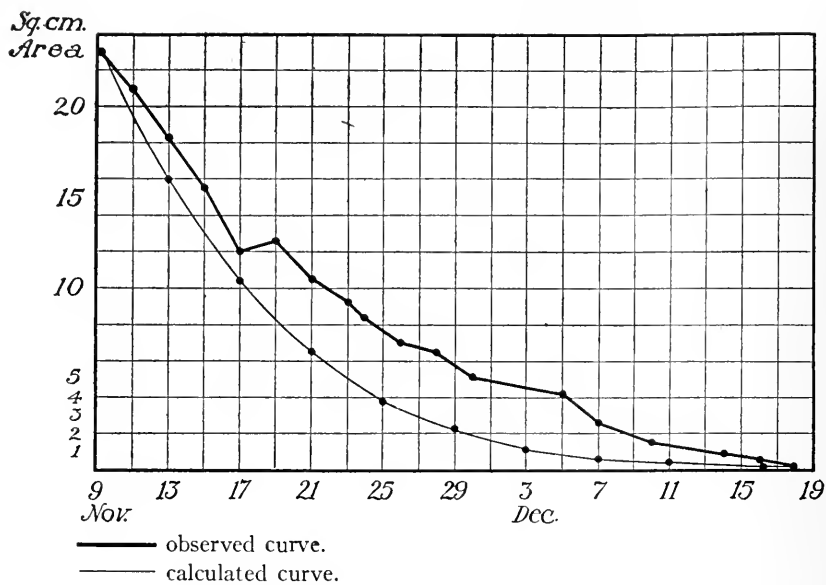
#### EXPERIMENTAL.

Surface wounds already covered with granulating tissue were selected. The normal rate of cicatrization of the wound was first obtained by sterilization with Dakin's hypochlorite solution or Daufresne's chloramine paste. The experiment was started as soon as the regular progress of the observed curve, as compared with the curve calculated according to du Noüy's formula, was established. The measurements of the wound and the plotting of the curve were made according to the technique previously described. Distilled water or hypertonic solution was applied to the surface of the wound by means of small perforated rubber tubes bound at the extremities and enveloped by a small cylindrical pad of Turkish toweling. The length of the pad varied from 4 to 8 cm., according to the size of the wound. Four threads placed transversely across the pad extended over the wound and were attached to the skin by means of small adhesive patches in such a manner as to keep the flushing tube in a fixed position over the granulations. The sterile liquid was contained in a flask placed about 1 meter above the patient's mattress, and reached the flushing tube by means of a Murphy drop tube. As a rule, in these experiments about 125 gm. of fluid flowed out per hour. Another technique was also used to bring into contact the surface of the wound and the distilled water or hypertonic solution. Agar cakes containing distilled water or hypertonic sodium chloride solution were applied to the surface of the wound. These constantly gave up their fluid contents, and were left at the surface of the wound, according to the nature of the experiment, from 6 to 12 hours and even 24 hours per day. In order to prevent immediate reinfection, the wound was flushed from four to six times in 24 hours with Dakin's solution. Another method consisted in applying for several hours every day a dressing composed of sodium stearate and chloramine paste. Chloramine paste, 10 parts per 1,000 was generally used, because reinfection takes place under 4 parts per 1,000. The wound

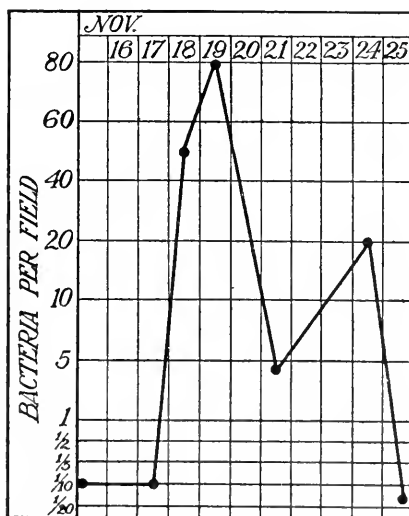
was examined bacteriologically every day. As soon as reinfection occurred the experiment was interrupted and the wound thoroughly sterilized. Every 4 days the surface of the wound was measured and the curve was plotted and compared with the calculated curve. The patients were kept in bed during the experiment and were under the supervision of a nurse day and night.

*Influence of Distilled Water on the Rate of Healing of a Sterile Wound.*

In the following experiments the wounds were flushed with distilled water from 2 to 4 hours every day.



Cicatrizization curve.



Bacteriological curve.

TEXT-FIG. 1. Experiment 1. Case 646.

*Experiment 1 (Text-fig. 1).—Case 646, age 25 years.*

Nov. 17, 1916. Sterile wound in the calf, 12 sq. cm. in area. The wound is surgically aseptic. Flushing with distilled water for 2 hours. Then flushing with 30 per cent hypertonic solution for 2 hours. The dressing was kept in place until the following morning.

Nov. 18. The appearance of the wound has not changed. Surface area 12.1 sq. cm. 30 to 50 bacteria per field. Same dressing. Flushing with distilled water for 2 hours. Flushing with a hypertonic solution for 2 hours.

Nov. 19. The appearance of the wound has remained the same. Surface area 12.6 sq. cm. 50 to 100 bacteria per field. Dressing with chloramine paste, 10 parts per 1,000.

Nov. 20. Same dressing. Surface area 11.1 sq. cm.

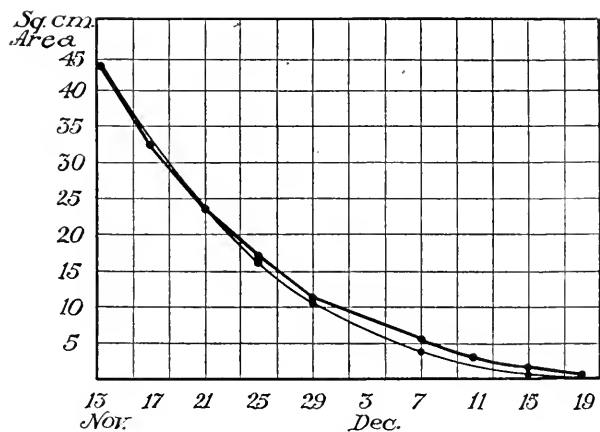
Nov. 21. Chloramine dressing, 4 parts per 1,000. Surface area 10.5 sq. cm. 4 bacteria per field.

Nov. 22. Flushing with distilled water for 4 hours. Chloramine dressing, 4 parts per 1,000 for 20 hours.

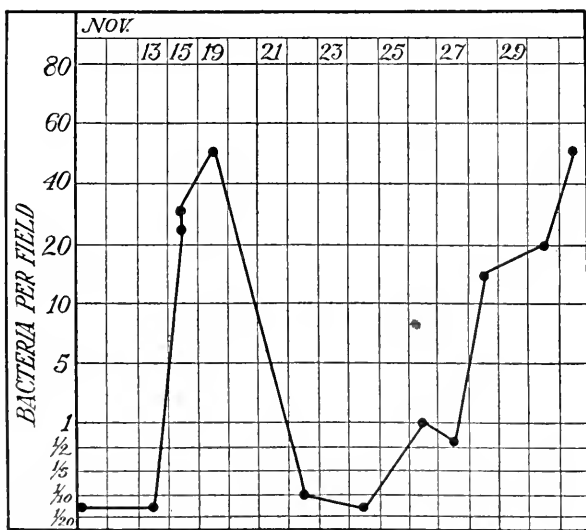
Nov. 23. Granulations smaller. Surface area 9.2 sq. cm. Flushing with distilled water for 4 hours. Chloramine dressing, 4 parts per 1,000.

Nov. 24. Same appearance. Surface area 8.4 sq. cm. 18 to 20 bacteria per field. Numerous cocci. Flushing with distilled water for 8 hours. Chloramine dressing, 10 parts per 1,000.

Nov. 25. 1 bacterium in 15 fields.



Cicatrizization curve.



Bacteriological curve.

TEXT-FIG. 2. Experiment 2. Case 694.

*Experiment 2 (Text-fig. 2).*—Case 694, age 20 years. Sterile wound 33.5 sq. cm. in area.

Nov. 17, 1916. Flushing with distilled water for 2 hours, followed by 2 hours' flushing with 30 per cent hypertonic sodium chloride solution. The dressing was not renewed until the following morning.

Nov. 18. The wound appears the same. Surface area 30.4 sq. cm. 20 to 30 bacteria per field. Flushing with distilled water for 2 hours and hypertonic solution for 2 hours.

Nov. 19. Same appearance. Owing to the infection, the rate of cicatrization has diminished. The surface area is 29.6 sq. cm. 55 bacteria per field. Chloramine dressing, 10 parts per 1,000.

Nov. 20. Same appearance. The curve has caught up with the normal curve. Surface area 26 sq. cm. 20 bacteria per field. Chloramine paste, 10 parts per 1,000.

Nov. 21. Same appearance. Surface area 23.6 sq. cm. 4 bacteria per field. Chloramine dressing, 4 parts per 1,000.

Nov. 22. Chloramine dressing, 4 parts per 1,000. 1 bacterium per 8 or 10 fields.

Nov. 23. Same treatment. Surface area 17.3 sq. cm. The wound is sterile and the experiment with distilled water alone can be resumed.

Nov. 24. Flushing with distilled water for 8 hours. Dressing with chloramine paste, 4 parts per 1,000. 1 bacterium in 10 to 15 fields.

Nov. 25. Flushing with distilled water for 8 hours.

Nov. 26. Flushing with distilled water for 24 hours. In order to keep the wound sterile, an injection of Dakin's solution is made every 6 hours. Surface area 14.6 sq. cm.; calculated area 14 sq. cm. 1 bacterium per field.

Nov. 27. Flushing with distilled water for 24 hours. 1 bacterium in 3 fields.

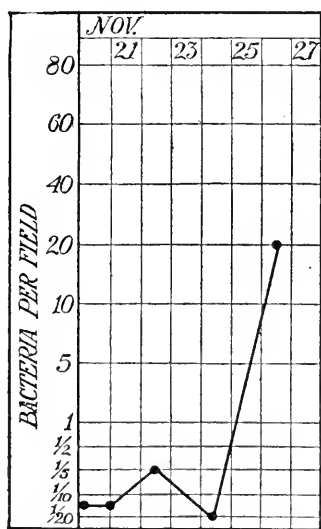
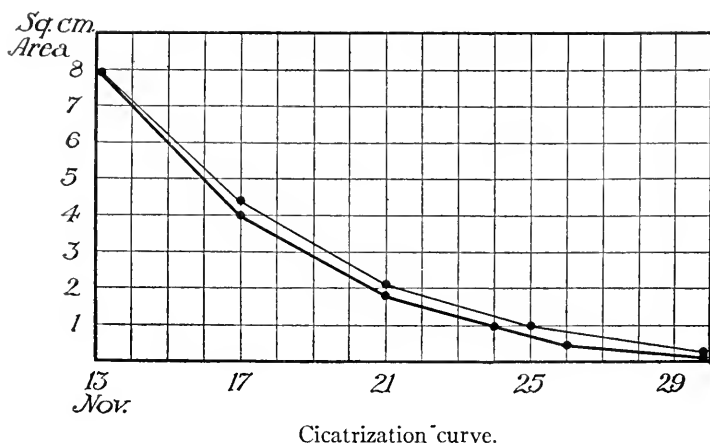
Nov. 28. Flushing with distilled water for 24 hours. Observed area 12 sq. cm. No apparent change. Calculated area 11.8 sq. cm. 10 to 15 bacteria per field.

Nov. 29. Flushing with distilled water for 24 hours.

Nov. 30. Retardation of the healing process. Observed area 11 sq. cm.; calculated area 9 sq. cm. 15 to 20 bacteria per field. Flushing with distilled water for 24 hours.

Dec. 1. Experiment interrupted on account of infection. Application of compresses soaked in Dakin's solution; renewed six times in 24 hours.

Dec. 2. Same dressing. Innumerable small bacilli.



TEXT-FIG. 3. Experiment 3. Case 694.



*Experiment 3 (Text-fig. 3).*—Case 694, age 20 years.

Nov. 21, 1916. Sterile wound of the wrist, measuring 1.8 sq. cm. Since Nov. 13 the process of repair has been regular. Flushing with distilled water for 2 hours, followed as in the previous experiment by application of chloramine paste, 4 parts per 1,000, in order to prevent infection.

Nov. 22. Flushing with distilled water for 4 hours, followed by chloramine dressing. Observed surface 1.5 sq. cm.; calculated surface 1.8 sq. cm. 1 bacterium in 4 fields.

Nov. 23. Flushing with distilled water for 6 hours. Dressing with chloramine paste.

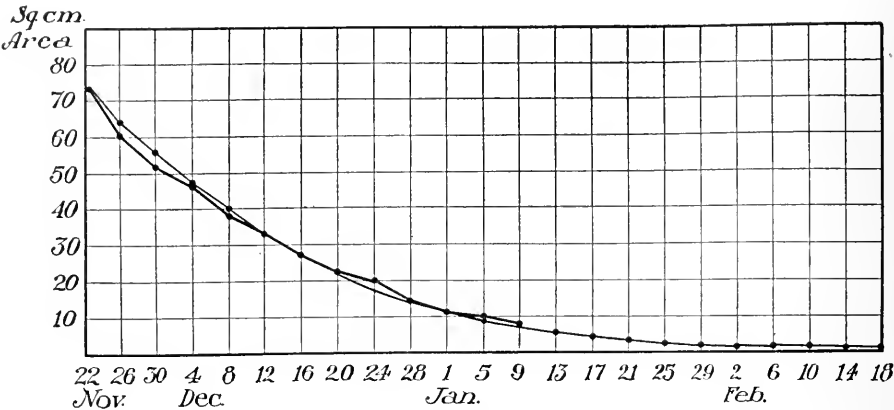
Nov. 24. Flushing with distilled water for 8 hours. Dressing with chloramine paste. 1 bacterium in 15 to 20 fields.

Nov. 25. Scale formed on the wound. Dry dressing.

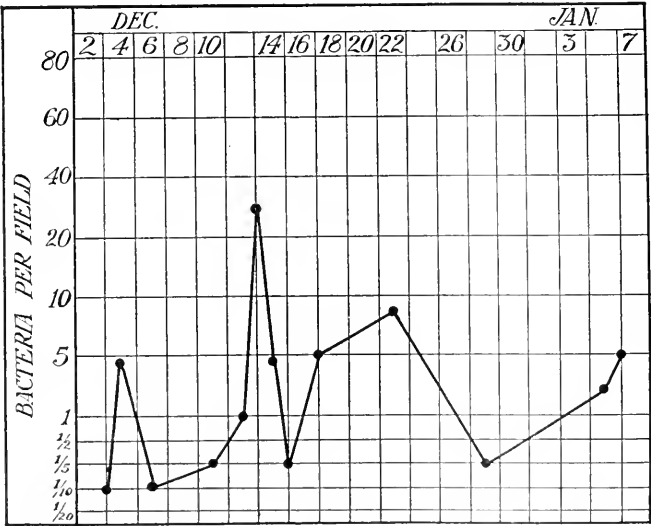
Nov. 26. Scale is removed. Area of the wound is 0.37 sq. cm.; calculated surface 0.7 sq. cm. 15 to 20 bacteria per field. Dressing with chloramine paste.

*Influence of Hypertonic Sodium Chloride Solution on the Rate of Healing of a Sterile Wound.*

In the following experiments the wounds were flushed with hypertonic sodium chloride solution.



Cicatrization curve.



Bacteriological curve.

TEXT-FIG. 4. Experiment 4. Case 639.

*Experiment 4 (Text-fig. 4).—Case 639, age 36 years.*

Dec. 1, 1916. The wound is practically sterile. 1 bacterium in 10 to 12 fields.

Dec. 2. Flushing with 40 per cent sodium chloride solution for 6 hours. Four injections with Dakin's solution. Surface area of wound 48.5 sq. cm.

Dec. 4. Flushing with 40 per cent sodium chloride solution for 12 hours. Four injections of Dakin's solution. Observed surface 46.5 sq. cm.; calculated surface 47.3 sq. cm. 3 bacteria per field.

Dec. 5. Same treatment.

Dec. 6. Same treatment. 1 bacterium in 8 to 10 fields.

Dec. 7. Same treatment.

Dec. 8. Same treatment. Six injections of Dakin's solution, instead of four. Area 38.5 sq. cm.; calculated area 39.7 sq. cm.

Dec. 9. Same treatment.

Dec. 10. Flushing with 40 per cent sodium chloride solution for 24 hours. Six injections of Dakin's solution. 1 bacterium in 8 fields.

Dec. 11. Same treatment.

Dec. 12. Same treatment. Surface of wound 33 sq. cm.; calculated surface 33 sq. cm. 1 bacterium per field. The curve as observed has finally overtaken the calculated curve and they now coincide exactly.

Dec. 13. Same treatment. 20 to 30 bacteria per field. The experiment is interrupted on account of infection.

Dec. 14. Sterilization of the wound by twelve flushings with Ringer solution.

Dec. 15. Same treatment. 1 bacterium in 6 fields.

Dec. 16. The experiment is resumed. A cake of agar-agar made of 40 per cent sodium chloride solution is applied to the wound during 12 hours. During the night the wound is sterilized by six flushings with Dakin's solution. Observed area 27 sq. cm.; calculated area 27.1 sq. cm. Sterile. The cake is applied for 9 hours.

Dec. 17. Same treatment. 4 bacteria per field.

Dec. 18. Same treatment. Surface observed 24.3 sq. cm.; calculated surface 24.2 sq. cm.

Dec. 19. A few bacteria.

Dec. 24. The wound is sterile. Agar cakes containing 50 per cent sodium chloride solution are applied during the day, and chloramine paste during the night.

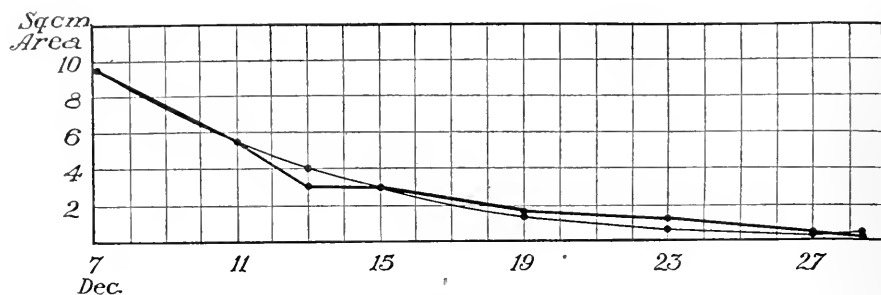
Dec. 28. The surface of the wound measures 14.2 sq. cm.; calculated surface 14.25 sq. cm. Same treatment.

Dec. 29. Same treatment.

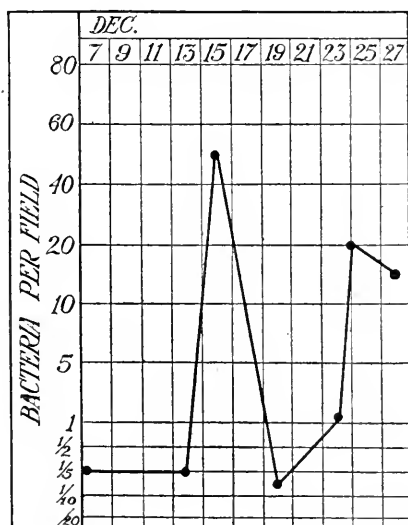
Dec. 30. Flushing with 80 per cent sodium chloride solution for 24 hours. Four flushings with Dakin's hypochlorite solution.

Jan. 1, 1917. Same treatment. Granulations congested. Small hemorrhages. Observed area 11 sq. cm.; calculated area 11.3 sq. cm.

Jan. 5. Same treatment with 80 per cent solution from Jan. 2 to 4. Observed area 10 sq. cm.; calculated area 9 sq. cm. 4 bacteria per field. The experiment is discontinued.



Cicatrization curve.



Bacteriological curve.

TEXT-FIG. 5. Experiment 5. Case 715.

*Experiment 5 (Text-fig. 5).—Case 715, age 23 years; Arabian. Leg wound.*

Dec. 7, 1916. Surface of wound 9.5 sq. cm. Dressing with Dakin's solution.

Dec. 11. Surface of wound 5.5 sq. cm.; calculated area 5.5 sq. cm. Flushing with 50 per cent sodium chloride solution for 24 hours, interrupted with two injections of Dakin's solution. Same treatment until Dec. 15.

Dec. 15. 30 to 50 bacteria per field. Four flushings with Dakin's solution in 24 hours. Surface observed 2.85 sq. cm.; calculated area 2.9 sq. cm.

Dec. 18. Flushing with hypertonic solution is substituted by the application of a cake of agar-agar containing 40 per cent sodium chloride. During the night three injections with Dakin's solution are made.

Dec. 19. Surface observed 1.5 sq. cm.; surface calculated 1.35 sq. cm. The injections are replaced by applications of chloramine paste, 10 parts per 1,000, the agar cakes being kept in contact with the wound for 12 hours every day.

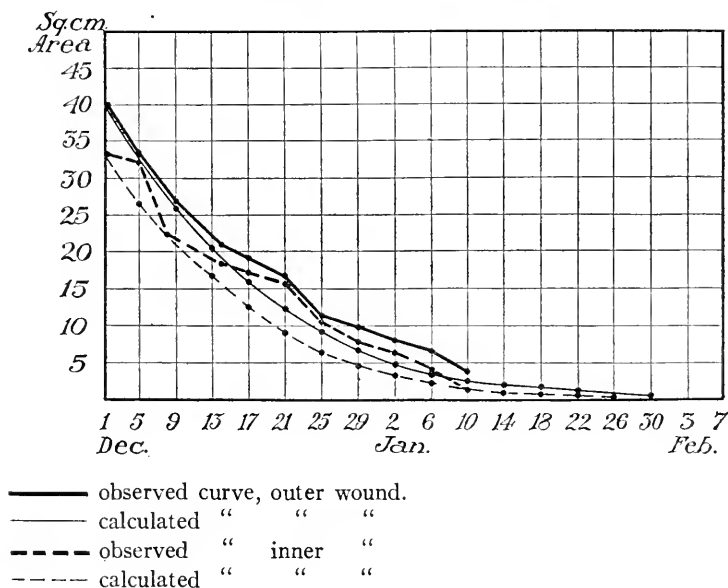
Dec. 23. Same treatment until Dec. 23. Observed area 1.1 sq. cm.; calculated area 0.6 sq. cm. The difference may be attributed to a slight infection. 2 bacteria per field.

Dec. 24. The 40 per cent agar cakes are replaced by a 50 per cent cake. The application of chloramine paste is continued during the night.

Dec. 27. Same treatment until Dec. 27, on which date it is calculated that healing will be effected. The wound, which is too small to be measured (less than 0.1 sq. cm.), is infected (10 to 15 bacteria per field), and this explains the retardation. Dressing with chloramine paste.

Dec. 29. The wound is completely healed.

*Comparison of Distilled Water and of Hypertonic Sodium Chloride Solution on the Same Patient.*



Cicatrization curve.

TEXT-FIG. 6. Experiment 6. Case 721.

*Experiment 6 (Text-fig. 6).—*Case 721. The patient has two wounds in the thigh, of about equal dimension, a small distance apart. The inner wound is flushed out with distilled water; the other with 40 per cent sodium chloride solution.

Dec. 3, 1916. Flushing of inner wound with distilled water for 12 hours. Four injections of Dakin's solution. Flushing of outer wound for 12 hours with 40 per cent sodium chloride. Four injections of Dakin's solution.

Dec. 4. 30 to 50 bacteria per field. Sterilization with Dakin's solution.

Dec. 8. Sterilization with Dakin's solution.

Dec. 12. Flushing for 24 hours per day under the same conditions described above, with six injections of Dakin's solution, continued until Dec. 16.

Dec. 17 and 18. Reinfection. Sterilization.

Dec. 20. Application to the external wound of an agar-agar cake, in the proportion of 40 per cent sodium chloride, and to the inner wound of a cake compounded with distilled water. 7 p.m. Dressing with chloramine paste. Same treatment until Dec. 27.

#### DISCUSSION AND SUMMARY.

In the study of the action of non-antiseptic substances on the rate of cicatrization, the chief obstacle encountered is the facility with which wounds become reinfected under an aseptic dressing. At the beginning of Experiment 1 the wound was sterile. It was subjected to flushing with distilled water for 2 hours, then to flushing with 30 per cent sodium chloride solution for another 2 hours. During that time no special precaution was taken to sterilize the wound and the dressing was left intact until the following morning. It was then found that the wound contained from 30 to 50 bacteria per field. The following day, after the wound had been subjected to the same treatment, the number of bacteria had increased to 50 and 100 per field, and as an immediate consequence the surface of the wound increased from 12 to 12.6 sq. cm. in 2 days. The wound was then dressed antiseptically and was found to be sterile 3 days later. Reinfection again took place the following day in spite of antiseptic dressing with chloramine paste 4 parts per 1,000, which was applied for 20 hours. In Experiment 2 similar results were observed. After 2 days of flushing with distilled water, the number of bacteria had increased to 50 per field. The wound was thereupon sterilized, but new reinfection ensued a few days later. Another wound on the same patient became reinfected under the same conditions after 1

day of sterile dressing. In none of the patients could the wounds be kept in a sterile condition throughout the whole experiment. It was impossible to maintain the sterility of a wound under aseptic dressing. Dakin's solution was therefore injected every 4 hours, or less often, according to the degree of infection, or chloramine paste was applied during the night. If there were 3 or 4 bacteria per field, the experiment was discontinued in order that the wound might be sterilized again. The cicatrization and bacteriological curves of Experiment 4 show that by the application of chloramine paste a wound may be maintained in an appropriately bacteriological condition for carrying out an experiment. Nevertheless, in spite of the antiseptic precautions taken, it was necessary to interrupt this experiment on two occasions, on December 13 to 15 and on December 18 to 22, in order that a complete sterilization of the wound might be effected. When the sterilization was performed as soon as the bacteria were discovered, little retardation occurred in the process of cicatrization. Moreover, the reinfection from the skin was often due to fine bacilli which have but mild retarding action on the rate of healing. The use of at least six flushings in 2 hours with Dakin's solution or of 12 hours' dressing with chloramine paste 10 parts per 1,000, was necessary to keep the wound in a condition of surgical asepsis.

The action of distilled water was studied in Experiments 1, 2, and 3. In Experiment 1 the wound was subjected to flushing with distilled water first for 2 hours, then 4 hours, and later for 8 hours per day. The wound was maintained in a condition of mild infection. No marked modification, either acceleration or retardation, was noted in the rate of repair during the period that the treatment was applied. From November 21 to 25 the wound was almost clean and the observed curve remained parallel to the calculated curve, showing that distilled water did not retard the rate of healing. In Experiment 2 the wound was subjected to uninterrupted flushing with distilled water, first for 2 and 8 hours, then for 24 hours. It was continued from November 24 to 30; *vis.*, for 112 hours out of 120, without the occurrence of any marked modification of the course of healing. The bacteriological curve showed that from November 22 to 27 inclusive the wound was kept aseptic. The slight retardation which occurred afterwards

was probably brought about by the infection. In Experiment 3 the wound was subjected to flushing with distilled water, first for 2, then for 4, 6, and 8 hours, a total of 20 hours in 4 days. From November 21 to 24 the wound remained surgically aseptic. No modification in the rate of healing occurred.

The action of the hypertonic sodium chloride solution was studied in a similar way. In Experiment 4 the wound was flushed at first with 40 per cent sodium chloride solution, from December 4 to 9 for 12 hours a day, and from December 10 to 13 for 24 hours a day, making a total of 144 hours out of 240 hours. At the end of this time the surface area of the wound coincided exactly with the calculated area. Owing to reinfection the experiment was suspended. From December 24 to 29 the wound was kept in contact with 50 per cent sodium chloride solution for 54 hours, and after December 30 flushing with 80 per cent solution for 24 hours a day was resorted to. The total amount of time involved in the above treatments was 174 hours with 40 per cent solution, 72 hours with 50 per cent solution, and 120 hours with 80 per cent solution. On January 1, the surface measured 11 sq. cm. and the calculated surface was 11.3 sq. cm. On January 5 the surface observed was 10 sq. cm. and the calculated surface was 9 sq. cm. It should be noticed that on January 5 the bacteria numbered 4 per field, which might account for the difference. In Experiment 5 the wound was flushed for 24 hours every day with 50 per cent sodium chloride solution from December 11 to 18, a total of 192 hours. From December 18 to 24 the wound was dressed with agar-agar cakes containing 40 per cent sodium chloride. The concentration was raised to 50 per cent from December 24 to 27. The cicatrization curve indicates only a slight retardation of the repair which can be attributed to infection when both cicatrization and infection curves are compared. The temporary acceleration on the 13th may have been due to the influence of the dressing, but as it did not occur again an experimental error is probably the cause of the change observed in the curve. In Experiment 6 two practically identical wounds at a distance of but a few centimeters from each other were located on the right thigh of Patient 721. The areas of the wounds were respectively 40 and 33 sq. cm. One of the wounds was flushed with distilled



water only. The other was subjected to the action of 40 per cent sodium chloride solution. From December 20 to 25 both wounds were in a condition of surgical asepsis. However, the cicatrization curves show that in spite of the difference of treatment the rate of healing was not modified.

The rate of healing of the wounds did not therefore apparently undergo any measurable modification under the influence of distilled water or hypertonic salt solution. It is well known that the osmotic changes of the medium have a marked influence on tissues deprived of circulation. But it seems that a tissue with normal circulation is protected by it against the changes of the osmotic pressure occurring at its surface. The above experiments show that apparently the conditions of the tissues of a wound are not modified by the changes of the osmotic pressure of the dressing. The beneficial effects of hypertonic sodium chloride solution on the sterilization of wounds and on the rate of healing recently described by various surgeons are possibly an illusion due to lack of precise technique.

#### CONCLUSIONS.

1. The flushing of an aseptic granulating wound with hypertonic sodium chloride solution or distilled water brings about an immediate reinfection.
2. Distilled water and hypertonic sodium chloride solution do not modify to a measurable extent the rate of healing of an aseptic wound.



# HUMAN PULMONARY DISTOMIASIS CAUSED BY PARAGONIMUS WESTERMANNI.

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PLATES 22 TO 31.

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## INTRODUCTION.

Pulmonary distomiasis is caused by *Paragonimus westermanni* Kerbert, and prevails extensively in the Far East. Ringer (1) discovered the parasite in 1879 in a patient at Tamsui in Formosa, and it has since been found in various parts of Japan by several observers. In certain localities it prevails as an endemic disease, and has for a long time aroused the interest of Japanese investigators. The development of the worm has, however, remained unknown except for the fact that the eggs are ejected with the sputum and that they hatch in water into free miracidia.

### *Prevalence of Pulmonary Distomiasis in Formosa.*

Although Ringer first found cases of pulmonary distomiasis in Formosa in 1879, the actual prevalence of the disease has remained unknown. In 1910 Nagano (2) reported that the disease prevailed in the northern part of Formosa around the Prefecture of Shinchiku. During 1913 and 1914 (3), I secured records of 1,249 cases, of which 922 occurred in the Prefecture of Shinchiku, the most thickly infected region on the island. I examined the pupils of all the public schools in the Prefecture of Shinchiku in December, 1914, and found that 4.3 per cent were suffering from the disease.

The morbidity among the young is less than among adults. However, if we assume this percentage to be that of the whole population of the Prefecture of Shinchiku, there are 13,000 cases in this region alone. The results of my observation differ from those of

Matsuo and Yokokawa (4) regarding the morbidity in Sansaka, Koryo, Jukirin, and Nansho. The discrepancy is probably due to the fact that the people of those villages have sunk wells and stopped drinking the river water during the period between their investigation and mine. The infection is more prevalent among people living along large rivers, such as the Hozankei, Komodenkei, Chukokei, and Koryokei. The inhabitants of the mountainous regions also were thought to suffer from this disease, but no investigation had been undertaken. In 1914, appointed by the Commission of Investigation of Formosan Endemic Diseases, I went into the districts inhabited by savage tribes and found that in the lowlands an enormous number of cases, *i.e.*, 50 per cent of the total population, was infected; in the highlands the cases seem to be less in number, although I was unable to carry out a thorough investigation there.

*Investigation of the Intermediate Hosts of Paragonimus westermanni.*

The Prefecture of Shinchiku and the villages of the savages in that vicinity are favorable districts for carrying out observations upon the parasites. As soon as I was appointed the Physician-in-Chief of the Government Hospital at Shinchiku, I began to search for the intermediate hosts.

As the first step the development of the eggs and the processes of hatching were studied, but efforts at experimental infection of animals both *per os* and subcutaneously with the miracidia were unsuccessful. Hence I concluded that at least one intermediate host functions prior to the infection of man. Various species of mollusks occurring in the local streams were examined microscopically, and seventeen different kinds of cercariæ were found, but it could not be determined which represented those of *Paragonimus westermanni*.

The next step was to ascertain which mollusks the miracidia of pulmonary distomas infest. For this purpose various kinds of fresh water mollusks were kept in water in which the miracidia had been made to hatch; and it was soon found that the miracidia prefer *Melania libertina* and *Melania obliquegranosa*. Attempts to keep the mollusks alive in aquaria failed. From the fact that the only species of fresh water mollusks that live in the thickly infected regions

is *Melania libertina* Gould, it was assumed that the cercariæ with a characteristic organ in the oral sucker<sup>1</sup> found in the snail must be the young of the pulmonary distomas. But how the miracidia find their way into the mollusks and develop into cercariæ has not as yet been ascertained.

Because of the difficulty of verifying this assumption experimentally an effort was made to discover the second intermediate host according to Kobayashi's (5) method employed in human liver distomiasis. This consists in infecting animals with the young distomas occurring in certain fish. After many attempts I found in Kalapai, in September, 1914, many encysted cercariæ in the liver and gills of certain crabs, which bore a striking resemblance to mature pulmonary distomas. I finally succeeded in discovering their path of penetration to the lungs of the final host. The facts that the cercariæ mentioned above enter the second intermediate hosts, *i.e.*, the crab, and that the cercariæ embedded in *Melania libertina* are the cercariæ of *Paragonimus westermanni*, have now been satisfactorily established.

#### *Development of the Worm within the Egg.*

Observations on the development of the worm within the egg have already been made by Nakahama, Manson, and Garrison and Leynes, in Japan, China, and the Philippines. The procedure that I employed for the Formosan form is as follows: A quantity of expectorated sputum containing the eggs is placed in a glass dish which is filled with water to the depth of 20 to 30 mm. The vessel is left uncovered in a dark place, and the water changed daily. The sputum and eggs sink to the bottom of the vessel and are readily examined microscopically. Too much sputum is to be avoided as the development of the egg is hindered; test-tubes are unsuitable as the worms do not hatch well.

The egg of *Paragonimus westermanni* in fresh sputum is oblong (0.063 to 0.084 by 0.045 to 0.054 mm.) and yellowish brown (Fig. 1). It has an operculum at one end and contains an embryo and several yolk cells (Fig. 2). The yolk cells gradually enlarge and the granules manifest Brownian movement which lasts until the yolk cells are completely taken up by the embryo. At the ten cell stage

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<sup>1</sup> These will be described fully below.

or a little later, the cell boundaries become indistinct. When the miracidium is about visible (Fig. 3), the yolk granules are diminished, and the contents of the egg become clearer. The rounded end of the ovoid embryo is directed toward the operculum and the oral part develops at that end (Fig. 4). At this stage the embryo resembles a melon seed and is covered with fine cilia. A few days later the embryo takes on a slow vermicular motion, becoming gradually more active until the miracidium bends upon itself. The cilia cover the surface of the body except the oral part, and are longest over the anterior part, especially about the protruding anterior end. They point posteriorly, except near the oral part where the direction is reversed. Through the vigorous movement of the cilia of the miracidium the operculum is torn off and the embryo is set free in the water (Fig. 5).

The egg containing the full grown miracidium measures 0.0792 to 0.09 by 0.0486 to 0.0567 mm., which is larger than that seen in the sputum. The mature miracidia measure 0.0612 to 0.072 by 0.036 to 0.045 mm.

The rate of development of the miracidia varies with the temperature and is retarded by cool weather. During the summer in Shinchiku, *i.e.*, from May to October, melon seed-like miracidia develop in 14 to 15 days, begin to move in 19 to 22 days, and hatch in 23 to 28 days. In March and April they take some weeks to hatch, and the miracidia remain for a long time within the egg even though they are as lively as in the warm season. From November to February or March no development was noted, though the eggs were watched constantly. According to my observations, the temperature for hatching is 25–31°C., and embryonic development ceases below 25°C. Manson (6) gives 26–34°C., Nakahama (7) 30°C., and Garrison and Leynes (8) 25–34°C. At 37°C. the eggs seem to disintegrate. The incubation period in summer is variable even in the same culture. Some eggs hatch in about 3 weeks, others as late as from 5 to 8 weeks. This difference may be due to other causes than temperature; possibly the length of time elapsing since the eggs were passed by the adult worm in the lungs of the patient before they were ejected with the sputum plays a part.

The miracidium when hatched measures 0.081 to 0.099 by 0.036 to 0.054 mm. It is provided with an alimentary tract and ganglion, two flame cells, and numerous granular embryonic cells. As it swims, the anterior end may not be pointed but invaginated. It can be kept alive for only a short time. In several hours its motion becomes sluggish and it assumes a ball-like appearance. Then it moves with a spiral motion and soon becomes inactive and dies.

Garrison states that direct sunlight is injurious to the miracidia. I also observed that embryonic development is arrested even in diffused light if this is intense. On the contrary, if the culture is kept in the dark, development goes on vigorously. Oxygen seems to be necessary for the development of the embryo. The vessel containing the eggs must be kept uncovered, and care should be taken

to change the water at least once a day. The eggs contained in sputum gradually become brown and die if dried, a space forming between the egg membrane and the shell. If the miracidia either within or without the egg membranes are subjected to 1 per cent hydrochloric acid, movement ceases and they die. It is known that they cannot resist the gastric juice.

In October, 1913, I introduced a quantity of water that contained full grown miracidia, either swimming free or still in the egg, into the stomach of pups; other pups were placed in the water containing free swimming miracidia. In neither case were the animals found to be infected by distomas, when killed and examined 45 to 100 days later. The miracidia appear to be unable to enter the animal body either *per os* or subcutaneously, unless they go through the second intermediate hosts.

*The First Intermediate Host and the Cercaria of Paragonimus westermanni.*

The cercariæ of *Paragonimus westermanni* were detected in the following three species of fresh water snails.

1. *Melania libertina* Gould (Fig. 20, a), which lives in pools or sluggish streams. In the villages of the savage mountain tribes, this species alone was found. Larger specimens than those seen in Shinchiku occur commonly in the main islands of Japan.

2. *Melania obliquegranosa* Smith (Fig. 20, b), which thrives in slowly flowing streams, is common in the flat region of Shinchiku.

3. *Melania tuberculata* Mueller, which occurs rarely in Shinchiku.

All the larger specimens of *Melania libertina* collected in the savage villages where pulmonary distomiasis occurs abundantly were found to be infected with the cercariæ. The occurrence of the cercariæ in *Melania obliquegranosa* collected in the lowlands parallels with the degree of infection. Cercariæ were found in only one specimen of *Melania tuberculata*, collected in Runasho village.

When the snails are placed in water containing miracidia, the latter swarm around them and become attached to the heads, jaws, and feet, and but rarely to the tentacles and mantles. They cling with their suckers, insert probosces into the tissues of the host and en-

ter the body of the snail like the cercariæ of *Schistosomum japonicum*, as described by Miyairi (9). Unlike the miracidia of *Schistosomum*, those of the pulmonary distoma shed their cilia in this act.

The cercaria measures 0.12 long by 0.09 mm. wide, and its tail is 0.054 mm. long. The oral sucker, 0.036 by 0.032 mm. in diameter, is provided with two pear-shaped bodies, the apices of which point posteriorly. It also has a spine, which appears to have a ring on its point. The posterior sucker is smaller than the oral one, having a diameter of 0.018 mm. The cercaria has three pairs of poison glands. The excretory vesicle of the glands is heart-shaped (Fig. 9).

Besides the cercariæ, sporocysts of various sizes are found abundantly in the liver of *Melania*. They are sometimes found in the heart and kidneys (Figs. 6 to 8, and 33).

The cercariæ mentioned have been detected in several specimens of *Melania* that thrive in the infected regions of the Prefectures of Niigata, Gifu, Tokushima, and Okayama on the main island of Japan.

The identification of the cercariæ with those of *Paragonimus westermanni* is based on the following data.

1. Only the cercariæ under consideration are found in the most thickly infected regions, among the savage tribes.

2. The miracidia of *Paragonimus westermanni*, which usually develop into the cercariæ described above, can enter *Melania* only.

3. The shape of the spine of the oral sucker of the encysted cercariæ in the crabs, and of the excretory vesicle of the young encysted cercariæ bears a striking resemblance to those of the cercariæ mentioned above.

4. The cercariæ successfully infected crabs free from distomas and developed into the specific encysted cercariæ.

The last fact seems to be the strongest evidence. The aquarium used for the experiment was a wooden box 3 feet long and 2 feet high. Three sides were completely covered by a thin cloth in order to prevent the intrusion of cercariæ from without. Of course, prior to the experiment it was ascertained that the cloth did not allow cercariæ to pass through. The cloth was covered by wire netting with meshes of  $\frac{1}{3}$  mm., in order to protect it from being torn by the crabs. The bottom of the box was covered with sand and pebbles. The aquarium was placed in a stream so that the crabs might be kept in as natural a condition as possible. Some snails that had been shown to contain the cercariæ were kept with the distoma-free crabs for a time. Non-infected crabs were difficult to obtain. I failed



to secure distoma-free crabs from the streams that run through Formosa, even in those regions where pulmonary distomiasis does not occur. Finally I succeeded in collecting young *Potamon (Geothelphusa) obtusipes* and *Potamon (Geothelphusa) dehaanii*, and fifty specimens of the former and twenty of the latter were examined and found to be free from the encysted cercariae. On September 4, 1915, the crabs and *Melania* were put together in the aquarium in the stream. Examinations were made daily. Most of the crabs climbed up the walls and were outside the water. Both the crabs and the snails began to die one after another. On October 10, 1915, I examined twenty specimens each of *Potamon obtusipes* and *Potamon dehaanii*. None of the former had encysted cercariae, but one of the latter showed a few, some of which had not been in the host for many hours. 3 weeks later thirty-five specimens of *Potamon dehaanii* were examined, and three were found to be infected by young encysted cercariae. The experiments are being continued.

Five young specimens of *Potamon obtusipes* and of *Potamon dehaanii* were placed in a vessel filled with water containing a large number of cercariae, obtained by crushing snails. In 3 days one of the former species was found to harbor a few encysted cercariae. It is evident that the infection occurred in the vessel; for the embedded, encysted cercariae were so young that they could not have been many hours in the host. The reason why so few crabs become infected experimentally may be due to differences in environmental conditions.

### *Crabs as the Second Intermediate Host.*

Seven species of fresh water crabs collected in the infected and non-infected regions were examined for encysted cercariae. The following three species contained them.

1. *Potamon (Geothelphusa) obtusipes* Stimpson (Fig. 21), which is the first species in which the encysted cercariae of *Paragonimus westermani* were found, and which has only been found in the mountainous regions of Shinchiku. It seems probable that this species is peculiar to the regions mentioned above, though Stimpson points out that specimens collected in the island of Amamioshima in the Prefecture of Kagoshima are identical with them.

I found this species abundant in the streams running through the mountainous regions in the Prefecture of Shinchiku, and also present in the mountainous regions of Usekiko village in the Prefecture of Taichu and Chikutoki village in the Prefecture of Kagi. The occurrence in this species of encysted cercariae corresponds with the degree of infection. Among the savages, where 30 to 50 per cent of the

population suffer from the infection, 80 to 100 per cent of this species harbored encysted cercariæ. Moreover, in Kalapai village, in which 55 per cent of the inhabitants suffer from pulmonary distomiasis, all the crabs harbored encysted cercariæ; while only 11 per cent of the crabs from the creeks of Naiwan, situated about 4 miles away, showed encysted cercariæ. The inhabitants of the latter village are believed to have the disease but rarely. In Sansaka, where distomiasis seldom occurs, the crabs showed no encysted cercariæ.

2. *Potamon (Geothelphusa) dehaanii* White (Fig. 22), which is found in the same locality, but less abundantly than the preceding species.

3. *Eriocheir japonicus* De Haan (Fig. 25) which, unlike the two species mentioned above, never occurs in the streams of the mountainous regions, but is found in the rivers flowing across the plain.

300 large specimens and over 30 small specimens were examined microscopically in February, 1915, for encysted cercariæ. Of these only two showed, in the gills, one encysted cercaria each. At first it was suspected that this was accidental, but later I learned that cercariæ may be found, though rarely, in the gills and muscles of this crab in the village of Torunsho.

Thus it seems to have been conclusively demonstrated that these three species of fresh water crabs act as the intermediate host of *Paragonimus westermani*; the first species showed the largest percentage of infection, the second a much smaller percentage, while the third was but rarely infected. The first species is found exclusively in Formosa, while the second and third are found also in other parts of Japan. I came, therefore, to the conclusion that these last two species play the part of the second intermediate host in the main islands of Japan.

Recently, Kobayashi (10), Ando (11), and Yoshida (12) demonstrated that the second species is the intermediate host in the infected regions in the Prefectures of Niigata and Gifu, and the third species in the Prefecture of Tokushima, and both the second and third species in the Prefecture of Okayama. Yoshida (12) claims that another species of crab, *Sesarma dehaanii* Milne-Edwards (Fig. 24), is the second intermediate host in the village of Hiyeshima in Nishinaru County in the Prefecture of Osaka. This species is also found in the region near the sea in the Prefecture of Shinchiku in Formosa, but no encysted cercariæ are

found in it. Moriyasu, Arima, and Tanakamaru (13) lately detected a species of crab that harbors encysted cercariæ, of which they kindly sent me specimens. Upon examination, they were found to belong to the third species described above. Professor Miyairi of the Kyushu Imperial University also found that *Astacus japonicus* DeHaan is the intermediate host of *Paragonimus westermanni* in Korea. In one of the infected regions in Formosa, i.e., in the village of Shinko, Prefecture of Taihoku, the third species as well as another variety, *Potamon (Parathelphusa) sinensis* Milne-Edwards (Fig. 23), was found by Yokokawa (14) to have encysted cercariæ. The following table shows the species and the localities in which they act as second intermediate hosts.

1. <i>Potamon (Geothelphusa) obtusipes</i> Stimpson.	Prefecture of Shinchiku.
2. <i>Potamon (Geothelphusa) dehaanii</i> White.	“ “ “
	Prefectures of Niigata, Gifu, and Okayama.
3. <i>Eriocheir japonicus</i> De Haan.	Prefectures of Shinchiku, Tokushima, and Okayama, and Korea.
4. <i>Sesarma dehaanii</i> Milne-Edwards.	Prefecture of Osaka.
5. <i>Astacus japonicus</i> De Haan.	Korea.
6. <i>Potamon (Parathelphusa) sinensis</i> Milne-Edwards.	Prefecture of Taihoku.

*Encysted Cercariæ of Paragonimus westermanni in Crabs.*

The size and shape of encysted cercariæ vary with their age. The younger ones are found embedded chiefly in the liver, while the older ones occur either in the liver, the gills, or even in the muscles. In the liver, they lie exclusively in the interspace of tissue.

*1. Youngest Forms.*—The youngest forms (Figs. 10 and 11) are not commonly detected in the crab, as the cercariæ usually enter at night. The youngest individuals present in the crabs were found at 5 o'clock in the morning just pushing their way into the parenchyma of the liver. They measured 0.13 by 0.05 mm. and had no tail. The posterior part is narrower than the anterior; they possess an oral sucker (0.04 mm. in diameter), and a spine in the oral sucker, which corresponds with that observed in the encysted cercariæ, except that it has no ring and a small posterior sucker (0.02 mm. in diameter) situated in the middle of the body. There is no excretory vesicle. In the anterior part of the body there is a tube-like organ which looks like the duct of poison glands. The worm appears as a white speck on the yellow parenchyma of the liver.

Immediately before transformation into encysted cercariæ the individuals are rarely seen, but not so rarely as the preceding stage. They now are folded on themselves and contract and extend their heads alternately but remain fixed

to the same spot. A little later a thin cyst, 0.11 mm. in diameter, encloses the worm, and within it the larva moves (Fig. 12). In still further advanced stages the cyst measures 0.13 mm. in diameter, the oral sucker is smaller, and a lumen (probably the pharynx) is seen immediately posterior to the latter. The posterior sucker has now enlarged slightly, while at the posterior extremity and along the median plane a rudimentary excretory vesicle, which sends off branches parallel to the axis of the body, has appeared (Fig. 13). Still later the cyst measures 0.14 mm. in diameter and there is a wider excretory vesicle, filled with coarse granules (Fig. 14). Finally, the larvæ lie straight within the cyst, the excretory vesicles gradually become heart-shaped, and fine dark granules appear and soon assume the form to be described.

2. *Young Cercariæ* (Figs. 15 to 18).—The young cercariæ possess a cyst measuring 0.18 to 0.2 mm. in diameter in which they lie extended. They have a large black excretory vesicle, and relatively large oral and posterior suckers, which tend to assume an ellipsoidal shape due to pressure. The oral sucker is 0.035 to 0.042 mm. long and 0.05 to 0.056 mm. wide, while the posterior sucker is 0.035 to 0.043 mm. long and 0.052 to 0.057 mm. wide. The spine of the oral sucker is present though it can be detected only with difficulty. No alimentary canal is differentiated. The cyst consists of chitin which is 0.005 to 0.006 mm. in thickness.

The young encysted cercariæ can be seen by the naked eye, in a piece of liver crushed between two slides, as small white dots lying between the lobules.

3. *Full Grown Cercariæ* (Figs. 19 and 34).—These are contained within a cyst from 0.26 to 1.0 mm. in length and 0.4 mm. in width. The oral sucker measures 0.07 to 0.09 mm. in diameter. The black-appearing intestinal canals are thick and wind on both sides of a large excretory vesicle. The posterior sucker (0.09 to 0.11 mm. in diameter) is a little larger than the oral one, and tends to be hidden by the excretory vesicle. The whole surface is covered with short cilia. The thick cyst, measuring 0.01 to 0.014 mm., is one of the characteristics of the species. The cercariæ move sluggishly within them. The liver usually contains three to four and never more than ten. The muscles contain few cercariæ; the gills contain the most. In the gills of one crab 97 full grown cysts were found. The cercariæ in this stage are detached from the gills and 20 per cent are found floating in the water. Under natural conditions they seem to leave the gills and drift along the streams, which thereby become a source of infection for human beings.

The above observations were made chiefly on *Potamon obtusipes*. In *P. dehaanii* and *Eriocheir japonicus* the development takes place in a similar way.

#### *Experimental Infection with the Encysted Cercariæ.*

In order to determine to what species the encysted cercariæ in the crab belong, tests were conducted on dogs. Pups born in the

regions in which pulmonary distomiasis does not occur were chosen, since dogs are liable to infection under natural conditions. Two pups were fed twice, on September 23, and October 10, 1914, with large quantities of the gills and livers of crabs containing encysted cercariæ. One of them died on December 9, 60 days after the last feeding.

Numerous cysts were found in the lungs. Each cyst showed two or three full grown distomas that contained no eggs in the uterus. They were 4 to 5 mm. long by 2 to 3 mm. wide. The other animal died on December 25, 75 days after the last feeding. Numerous cysts containing egg-bearing distomas were found in the lungs. The worms were 6 to 7 mm. long and 3 to 4 mm. wide, just one-half the size of the worms of spontaneous infection in the cat and dog and in man, but morphologically they were identical with *Paragonimus westermanni*.

The experiment was repeated on two pups from a non-infected region (December 26, 1914, to February 7, 1915) with identical results. On another occasion animals were given water containing the encysted cercariæ which had been detached from the gills of the crab, and adult distomas developed also in these animals.

#### *The Course of Penetration of the Cercariæ in the Final Host.*

Since the distomas appear to reach their destination in the final host by passing through the walls of the alimentary tract, the next step was to make clear their course. Microscopical examination of the viscera of the infected animals indicated that the distomas passed through the walls of the alimentary tract and the diaphragm, and thus reached the lungs. Yamagiwa (15) believes that the distomas reach the lungs by way of the liver, and then by the diaphragm, the pleura, etc., since adherent nodules involving these organs have been demonstrated. My observations were made in the early stages, so that congestion and hemorrhage of the mucous membrane of the intestine could be detected as well as petechiæ in the diaphragm and lungs, especially the lower lobes. By studying the petechiæ, points were found through which the worm seemed to have passed. At this early period I concurred with Yamagiwa's views. Just at

this juncture Yokokawa (14) discovered many distomas floating in the serous exudates of the abdominal and thoracic cavities of dogs to which encysted cercariæ had been fed.

At the suggestion of Dr. Miyajima of the Kitasato Institute, I examined a dog and a cat sent from Shinchiku where they had been fed encysted cercariæ, for more than 10 days, and succeeded in determining the path of penetration.

*Experiment 1.*—A pup was fed from April 15 to 27, 1915, large numbers of crabs gathered in the savage villages. The animal died on April 27. The body cavity was opened and the animal immersed in 10 per cent formalin.

Macroscopical examination showed petechiæ in the mucous membrane of the intestine near the jejunum. The other abdominal viscera and the diaphragm presented no changes. The lungs showed general pneumonic areas. Since this condition was not met with in other cases it must be regarded as a complication.

Nearly the whole omentum was sectioned, and several worms were found: one (0.22 by 0.14 mm., smaller than usual owing to the fixation) in the parenchyma of the omentum near the greater curvature of the stomach, another (0.36 by 0.24 mm.) in the fat tissue, and others in the diaphragmatic ligament where the worms (0.3 by 0.18 mm.) were surrounded by hemorrhage. No worms were found in the liver or the mediastinum, and none were detected in the lungs. Similarly, the contents of the lymphatic system, the thoracic cavity, and the heart were negative.

*Experiment 2.*—A cat was fed for 15 days with a large number of encysted cercariæ (April 18 to May 2, 1915), when it was chloroformed, the body cavity opened, and the animal placed in formalin.

On macroscopical examination petechiæ of various sizes, some as small as 1 mm. in diameter, were found in the mucous membrane of the jejunum (Fig. 26). A note by the collector of the material stated that four or five grayish white vesicles were present on the diaphragm, which undoubtedly were worms. Small petechiæ were present in the capsule of the spleen, and in the muscle of the diaphragm. The surface of the lungs, especially the lower lobes, showed many petechiæ.

Sections were made through the basal part of the omentum, the liver, the ligament and tendinous part of the diaphragm, and the mediastinal tissues, but no worms were found. Large portions of the lungs were sectioned and worms were found entering the pleura on their way to the parenchyma of the lungs. In sections made through the jejunum a worm (0.28 by 0.18 mm.) was found that had just entered the external layer of muscle. This path was indicated by the torn muscle fibers, the infiltrated round cells, and especially the eosinophil cells (Fig. 35). No worms were found in the cœlom; hence they must have dropped when the material was fixed. Worms could not be detected in the act of piercing

the lungs. However, in an experimental animal 7 weeks after the feeding, a worm (2.2 by 1 mm.) that had already reached the parenchyma of the lungs was found. Thus, it may be said that the path by which the worm reaches the lung parenchyma has been ascertained.

In brief, the results of my observations support Yamagiwa's view; for they establish the point that cercariæ of *Paragonimus westermanni* taken *per os*, with the food, pierce the intestinal wall, pass into the abdominal cavity, then through the diaphragm into the thoracic cavity, and finally reach the lungs, where they produce cysts and grow into adults.

Since my discovery, Yokokawa (16) has also reported in several papers, published in Japanese, minute observations on the same subject, and they have been further supported by Kobayashi (10) and Ando (11).

Additional experiments were carried out in Formosa, of which illustrative protocols are here given.

*Experiment 3.*—From April 18 to May 16, 1915 (29 days), a kitten<sup>2</sup> was fed with many encysted cercariæ and then killed for examination.

The mucous membrane of the intestines was found to be congested, and several petechiæ were seen in the jejunum. Several worms were attached to the omentum. The liver was hyperemic, and the upper portion in apposition with the diaphragm was perforated by small pores that reached the parenchyma, in which young distomas were seen wriggling in and out. Many younger ones were also attached in the same region. Several worms were attached to the liver and the ligament of the diaphragm, while some were seen crawling on the lower surface of the diaphragm itself (Fig. 28). The capsule of the spleen presented several cyst-like protuberances and some petechiæ. The kidneys were normal. By washing the abdominal cavity with saline solution, sixteen young distomas were obtained. From the liver and the diaphragm, eleven worms were obtained.

In the thoracic cavity about 30 cc. of bloody turbid fluid were present, which contained one worm. The parietal pleura showed many cysts and petechiæ. On the thoracic aspect of the diaphragm many punctures and hemorrhagic spots were seen and some worms were present in the muscle layer (Fig. 36).

*Experiment 4.*—From April 28 to May 16, 1915 (19 days), a kitten<sup>2</sup> was fed on crabs collected among the savages.

The surface of the liver and diaphragm showed numerous perforations in which were young wriggling worms. The muscular layer of the diaphragm was the

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<sup>2</sup> These two animals were examined by my assistants in the Shinchiku Hospital during my stay in Tokyo.

seat of several hemorrhagic spots, as was also the visceral pleura. Thirty-six worms were found in the coelom, and thirty-five on the surface of the liver.

*Experiment 5.*—100 mature encysted cercariæ were fed *per os* to a kitten at 10 a.m. and 4 p.m. on June 26, 1915. The animal was killed 6 hours later.

There were about 20 cc. of light yellowish fluid in the abdominal cavity; no worms were found. In the upper part of the ileum a young distoma that had just come out of the cyst, and another still in the cyst, were found; in the jejunum were found numerous specimens of *Ascaris mystax*. The other organs were normal. The fluke found in the ileum measured 0.35 by 0.21 mm. The empty cyst measured 0.28 mm.

*Experiment 6.*—A kitten was fed with 100 full grown encysted larvæ at 3 p.m. on June 27, 1915, and killed for examination at 2 p.m., July 17; that is, 3 weeks after the feeding.

The animal was somewhat emaciated. The abdominal cavity contained a small quantity of cloudy fluid; no worms were found. The serous membrane of the intestine was congested. One worm was attached to the surface of the liver. Three worms were seen moving on the abdominal surface of the diaphragm. A worm was found at the greater curvature of the stomach, but no worms were free within the abdominal cavity.

The thoracic cavity contained a large quantity of cloudy reddish fluid from which twenty worms were obtained. Two worms were attached to the heart. Many worms were found on the pleura where the sternum comes in contact with the pleural diaphragm. The lungs presented a light reddish color with a few scattered hemorrhagic spots. In the central portion of the lower lobe of the left lung one grayish cyst as large as the tip of the small finger was found. One or two worms were seen intruding into the pulmonary pleura, with here and there cyst-like protuberances.

The worms in the thoracic cavity and in the abdominal cavity are identical. The largest specimen measured 2.2 by 1.4 mm., and the smallest 1.1 by 0.8 mm.

*Experiment 7.*—A kitten was fed 200 full grown encysted larvæ at noon on July 1, 1915, and died at 6 p.m. on July 19, 19 days after the feeding. Most of the encysted larvæ fed were taken from the gills of somewhat decomposed crabs.

Emaciation; the abdominal cavity contained a small quantity of fluid. The intestinal serosa was slightly congested. One worm was attached to the upper parietal pleura; two worms were found on the liver, one attached to the serosa of the stomach, and another to the serosa of the duodenum. A very small worm was free within the abdominal cavity. The surface of the liver showed a number of vermicular scars. Two worms were penetrating the capsule of Glisson. One worm was attached to the esophagus. In the right lung were seen three somewhat large hemorrhagic spots. Within the pleural cavity opposite the sixth right intercostal space within a hyperemic area a worm lay wedged (Fig. 30).

No difference in size was noted between the worms in the thoracic and in the abdominal cavities. The largest specimen measured 1.5 by 0.7 mm., the smallest 1.0 by 0.6 mm.



*Experiment 8.*—A pup was fed with 227 full grown encysted larvæ at 8 p.m., July 8, 1915, and died at about 2 a.m., July 14, or 126 hours after the feeding.

A large quantity of light yellowish fluid containing two or three worms was found in the abdominal cavity. The intestines showed here and there ulcerations in the mucous membrane and innumerable *Ankylostoma ceylanicum*. The stomach contained numerous ascarides. The liver showed two or three hemorrhagic spots, and similar areas occurred in the muscular layer of the diaphragm.

The thoracic cavity contained a large quantity of light yellowish fluid and a few worms. The lungs appeared pale, and a few hemorrhagic spots were seen. The worms in the abdominal and thoracic cavities were generally small, measuring 0.4 to 0.5 mm. by 0.2 to 0.3 mm.

*Experiment 9.*—A pup was fed fifty full grown encysted larvæ at 7 a.m., July 9, 1915, and died August 2; that is, 25 days after the feeding. The abdominal cavity was free from fluid. One distoma was attached to the upper left quadrant of the parietal peritoneum. The liver was hyperemic and showed a few serpentine scars on the surface. One worm was attached to the omentum. A few hemorrhagic spots were seen in the muscular layer of the diaphragm.

In the right thoracic cavity was a small quantity of bloody turbid fluid; there was none in the left. On the abdominal aspect of the diaphragm one active worm was seen, and another in the diaphragmatic ligament. The lungs showed numerous hemorrhagic spots but no cysts (Fig. 31).

The largest worm measured 3.0 by 1.2 mm., and the smallest 1.2 by 0.6 mm.

*Experiment 10.*—On September 9 and 10, 1915, a pup was fed 30 and 40 full grown encysted larvæ respectively, collected in a savage village. The animal was killed 45 days after the last feeding.

No fluid was present in the abdominal cavity. One worm was found on the abdominal surface of the diaphragm and another on the diaphragmatic surface of the liver. Both were small.

No fluid was present in the thoracic cavity. One worm was attached to the mediastinal tissues, and one to the pericardium. The lungs were reddish purple in color, and showed numerous dark brownish red hemorrhagic specks and gray cyst-like scars. The right lung contained about ten cysts, the left only one.

The largest worm measured 4.5 by 2.2 mm.; and the smallest was about one-half that size.

*Experiment 11.*—In order to determine whether the hairy crabs are the intermediate host of the worm, a pup was fed 200 crabs between July 27 and August 23, 1915, after the carapace and the legs had been removed, and the viscera and the muscles minced. The animal died at midnight on August 24. The crabs were bought in the market of Shinchiku and given to the animal without examination for the presence of the larvæ.

On the surface of the liver were one or two small hemorrhagic spots. In the right thoracic cavity was a small quantity of bloody fluid. One worm was attached to the lower lobe of the right lung, and one was seen crawling over the thoracic surface of the diaphragm. The lungs were pale with occasional large

dark bloody flecks, some of which had a wound in the central part where the parenchyma had been injured. In the inferior lobe of the right lung was seen a small dark red nodule, which had a small cavity in the center from 5 to 7 mm. in diameter and 12 or 13 mm. deep, at the bottom of which a worm was found.

In the ligament of the diaphragm were seen three small perforations the size of a pin-head. In the muscular layer of the diaphragm one or two hemorrhagic spots were present.

The worms in the thoracic cavity measured 3.0 by 1.5 mm., while those in the abdominal cavity measured 2.4 by 1.2 mm.

This experiment indicates that the hairy crabs may also act as the second intermediate host of *Paragonimus westermanni*.

Eighteen animals were employed<sup>3</sup> in the experiments, eleven of which are reported above.

The animals were given *per os* a comparatively small number of encysted larvæ, usually about 100. There developed in consequence mild infections with slight pathological changes.

Table I gives the results obtained.

TABLE I.

*Results of Feeding Encysted Larvæ of Paragonimus westermanni to Kittens and Pups.*

Experiment No.	Species.	Time between feeding and examination.	No. of worms.	Size of worms.
				mm.
1	Kitten.	6 hrs.	2	0.35 × 0.21
2	"	16 "		
3	"	1 day.	2	0.3 × 0.15
4	"	2 days.	1	0.73 × 0.21
5	"	2½ days.	1	0.5 × 0.22
6	Pup.	3 days, 15 hrs.	2	0.53 × 0.33 0.51 × 0.3
7	"	5 " 6 "	5-6	0.4-0.5 × 0.2-0.3
8	Kitten.	14 "	8	0.73-1.47 × 0.44-1.1
9	"	18 "	11	1.5-1.0 × 0.7-0.6
10	"	21 "	Over 25.	2.2-1.1 × 1.4-0.8
11	Pup.		4	3.0-1.2 × 1.2-0.6
	"	25 "	10	(No measurement taken.)
12	" *	45 "	4	4.5 × 2.2
			(Besides numerous cysts.)	

\*Experiments in which the animals were kept longer than 45 days after the feeding are described elsewhere.

<sup>3</sup> With the exception of Experiments 1 to 4 and 11, cercariæ were given to the subject only once.

The following conclusions have been reached. Encysted larvæ taken *per os* by the final host reach the jejunum, where they emerge from the cysts and make their way through the intestinal epithelium into the submucosa (Figs. 26 and 35). From that structure they pierce the muscular layer and pass into the abdominal cavity. This process, required, in the earliest case, 24 hours. Some larvæ remain in the intestines for 10 hours or more. Once in the abdominal cavity the worms proceed upward along the mesentery and the omentum and reach the liver (Figs. 27, 29, and 37), where they become attached to the diaphragm which is pierced at the ligament or in the muscular region (Figs. 28 and 36). The first worms pass into the thoracic cavity 77 hours after reaching the alimentary canal, but most of them remain on or in the liver for some time before they reach the thoracic cavity.

Some fail to reach the lungs, and, as Yokokawa believes, remain in the mesentery, the omentum, and the seminal ducts, forming cysts. In the thoracic cavity they often remain in the subvisceral pleural region or in the serous fluid. While in the pleural cavity they seem to grow before they enter the lung parenchyma where sooner or later they form cysts. It is doubtful whether they remain in the pleural cavity for a long time without forming cysts.

The changes in the lungs may be summarized as follows (Figs. 31, 32, and 38). 3 days after the feeding, a few pin-head hemorrhagic spots appear in the lungs. They are the beginning of wounds caused by the worms. In about 14 days, some of these spots become dark red, and in 21 to 25 days pale cysts have developed. Usually the cysts correspond in position to the petechiæ. The following phenomena have also been observed. In some instances, 45 days after feeding the encysted cercariæ, on the surface of the lungs numerous small dark red cysts are formed about which are infiltrations of polynuclear leukocytes or round cells in addition to the red corpuscles. In 50 days the surface of the lungs comes to have numerous dark reddish hard cysts as large as the tip of the little finger. The cut surface of the cyst appears dark red, from a previous hemorrhage; in the center is a vacuole the size of a pea. The distomas when present lie in the vacuole; but empty ones are also observed, for the worms may escape into the neighboring tissues. After about 90

days, the cysts have become bluish gray. On section a vacuole as large as a bean is found, in which porridge-like matter or one or two mature distomas are seen. In the latter case one of the two may be found dead. The walls of the cyst are composed of a thin layer of connective tissue.

The cyst wall may in some regions be wanting, the lumen being connected directly with the air spaces, a bronchus, or radicle of the pulmonary vein. Thus it is seen how the regional bleeding or pneumonic infiltration of the alveoli originates. Eggs were observed in worms 90 days after the feeding. When they are shed, they lie sometimes in the cysts, but oftener in the parenchyma of the lungs, mingling with the erythrocytes and leukocytes, epithelial cells of the lungs, or cellular detritus, etc. The bronchi and bronchioles near the lesions are dilated and contain erythrocytes or leukocytes together with eggs. The microscopical changes described indicate that the worms do not always remain within the cysts, but may emigrate into other parts of the lungs.

*Development of Cercariæ in the Final Host.*

The size of the distoma depends upon (a) the initial size of the cercariæ and (b) the nutriment. The following figures give the measurement of specimens fixed with alcohol.

TABLE II.  
*Size of Paragonimus westermanni at Various Ages.*

Age.	Length.	Breadth.
<i>days</i>	<i>mm.</i>	<i>mm.</i>
Just hatched.	0.3 -0.48	0.18-0.23
3	0.44-0.48	0.23-0.26
6	0.44-0.53	0.26-0.32
14	0.65-0.95	0.37-0.61
18	0.8 -1.6	0.4 -0.85
21	1.3 -2.0	0.8 -1.1
25	0.9 -2.2	0.7 -1.3
45	2.5 -3.5	1.5 -2.0
60	4.0 -5.0	2.0 -3.0
90	6.0 -7.0	3.0 -4.0

Young worms (Figs. 39 to 42) have relatively large suckers, as shown by the measurement of fresh specimens. The larvæ which have just left the cysts present an oral sucker whose diameter is 0.07 to 0.09 mm. and a ventral sucker 0.09 to 0.11 mm. in diameter. In a 14 day old specimen (*i.e.*, after the feeding) the oral sucker is 0.14 to 0.23 mm. and the ventral sucker 0.16 to 0.26 mm.; in 21 day old specimens the oral sucker is 0.22 to 0.33 mm. and the ventral sucker 0.23 to 0.36 mm.; in those 45 days old the oral sucker is 0.3 to 0.5 mm. and the ventral 0.33 to 0.53 mm. in diameter.

The encysted cercariæ have a large excretory vesicle, occupying nearly the whole central body space, which is filled with a black granular substance. In the final host the contents escape and the vesicle becomes a small dark-appearing space.

The development of the genital organs takes place slowly. In a young worm just out of the cyst, the gonads are hardly visible. In a 2 week specimen gonidial regions appear which are deeply stained by borax carmine. In 18 to 21 day specimens the differentiation of the ovaries and the uterus has taken place. The ootypes appear as small groups of cells, one on each side of and posterior to the ventral sucker. The uterus is a simple winding tube. The testes make their appearance at a region corresponding to the ovaries. In a 25 day specimen (Fig. 42) both the ovaries and the testes have sent out several branches. In 50 day specimens (Fig. 43) the gonads are mature, but the yolk glands are not yet fully developed. The 90 day specimens (Fig. 44) lay eggs and have mature yolk glands. To sum up, within 3 weeks the rudimentary genital organs make their appearance and within 3 months they begin to function.

#### *Resistance of the Encysted Cercariæ to Environmental Influences.*

It is of importance for the prevention of pulmonary distomiasis to determine the power of resistance of encysted cercariæ to external influences.

Young encysted cercariæ are so delicate that they die in a few hours after becoming detached from a crab. They never develop in the final host, a fact experimentally proved by feeding them to a dog.

If full grown encysted cercariæ are artificially removed from the gills of the crabs and put into clear water, they soon swell and curl within the cysts similarly to other distomas. If the cercariæ are small, they remain inactive for a day or two or even longer within the cysts. Encysted cercariæ kept in clear water for almost 3 days either remain curled up or develop a remarkably enlarged excretory vesicle and compressed intestine. None wriggled, but one or two dead worms were seen to have shed the cysts. On another occasion

the encysted cercariæ still attached to the gills were put into the water and allowed to stand for some time at room temperature (about 30°C.). After 3 days the results were as shown in Table III.

TABLE III.  
*Behavior of Encysted Cercariæ in Water.*

No. of gills.	Total No. of encysted cercariæ.	Empty cysts.	Dead cysts.	Living cysts.	Free cercariæ.
1	16	7	3	6	1
2	6	3	1	2	—
3	7	2	1	4	1
4	3	1		2	1
5	3	1	1	1	—
6	7	5	—	2	1
Total.	42	19 (45.2%)	6 (14.3%)	17 (40.5%)	4 (9.5%)

From this table it is seen that about half the encysted cercariæ hatched, while the other half remained within the cysts although still alive. A few were found dead inside the cysts. The remainder of the cysts, reexamined 5 days later, were found either evacuated or dead.

As Kobayashi (5) has shown with *Clonorchis sinensis*, the encysted cercariæ of *Paragonimus westermanni* leave the cysts on being kept several hours in fresh water, and the empty cysts are often seen in the alimentary canal of the final hosts, and it may therefore be inferred that the cercariæ escape before the cysts are digested in the intestine. In the winter, however, when the temperature in Shinchiku is about 15°C., the cercariæ do not escape from the cysts even if kept in fresh water for 2 weeks or more.

The question arises whether the cercariæ would infect herbivorous animals since they are liberated without any external agency. Ando (17) reported the successful infection of white mice, guinea pigs, and rabbits, to which encysted cercariæ had been fed. I also made experiments upon mice, but have reached the conclusion that the infection seems more difficult to achieve in these small animals than in dogs and cats.

The newly hatched cercariæ do not resist even slight temperature changes.<sup>4</sup> In the summer in Shinchiku when the room temperature is about 30°C. they all die in 20 to 30 hours. As long as the encysted cercariæ remain in cold streams ready to enter the final host, they do not become liberated. It is of interest from the standpoint of the prevention of distomiasis that the free, living cercariæ may be swallowed by man with water or food without harm.

On the other hand, the full grown cercariæ are enclosed within cysts much thicker than those of any other known species. The cysts are impermeable to both paraffin and celloidin. The resistance was tested with results which may be stated as follows:

When the crabs containing them are roasted over the fire until the muscles turn white the cercariæ are killed. Heating the crabs in water at 55°C. for 5 minutes also destroys them.

Heating the encysted cercariæ, removed from the crab, in water at 45°C. for 37 minutes does not kill them, but after heating to 55°C. for 10 minutes or to 70°C. for 5 minutes, they are killed.

The encysted cercariæ survive in 1 per cent solution of sodium chloride for 3 hours, or in 10 per cent solution for 2 hours. They first shrink but are soon restored to normal when transferred to fresh water.

The encysted cercariæ contained on the crabs' gills were put in soya sauce. At the end of 30 minutes they were alive, but after 9 hours they were killed. 50 specimens of the larger encysted cercariæ which had been immersed in soya sauce for 2 hours were fed to a kitten which when killed 3 weeks later was found not to be infected.

Immersion in vinegar for 30 minutes does not kill the cercariæ, but immersion for an hour does.

The above experiments may be summarized as follows: The cercariæ are liberated under natural conditions, if the temperature is high enough. When hatched they have little power of resistance to injurious influences. Within the cysts, on the contrary, they are hardy and withstand for quite a time immersion in solutions of table salt, soya sauce, and vinegar. Hence, crabs are dangerous unless they have been in soya sauce or vinegar for 2 hours or more after their carapaces are removed. A solution of table salt at the con-

<sup>4</sup> This was confirmed by the experiments of Matsui, Y., Biological studies on the cercariæ of the pulmonary distoma. *Hokuyetsu Igakkwai Zasshi*, 1915, xxx, No. 3.

centration used for culinary purposes is not strong enough to kill cercariæ. Though the cercariæ are not especially resistant to heat, half boiled or half roasted crabs are unsafe as food.

*Principal Causes of the Prevalence of Pulmonary Distomiasis  
and Its Prevention.*

It still remains to be determined how human pulmonary distomiasis is caused. At first I entertained the notion, in conformity with that of Ando (11) and Moriyasu, Arima, and Tanakamaru (13), that the encysted cercariæ, separated from the gills, which survive for a time on the surface of the water and which may be found free in water in which infected crabs have been, are the sources of infection. But later I concluded that the eating of crabs containing the cercariæ is the chief cause of the disease. Of twenty-two patients suffering from the disease, seventeen, or 77 per cent, gave a history of eating hairy crabs and three, or 13.6 per cent, red crabs.

An objection to this view may be found in the fact that only about 0.5 per cent of the crabs are infected. But a similar condition is found in liver distomiasis, for Kobayashi (5) has affirmed that the least infected fish, *Carassius auratus*, is responsible for most of the hepatic distomiasis. This view is supported by the statement that raw crabs are eaten in some parts of Japan and particularly in the highly infected regions of Korea.

Hence it may be concluded that the eating of raw or imperfectly prepared crabs and the drinking of river water which they inhabit are the two principal causes of pulmonary distomiasis.

CONCLUSIONS.

1. The morbidity of pulmonary distomiasis among the school children in the plains of the Prefecture of Shinchiku is 4.3 per cent, while in the mountainous regions among the savages it reaches in some districts 50 per cent.

2. Seventeen species of cercariæ were discovered in fresh water mollusks in the Prefecture of Shinchiku, Formosa. But it was impossible to ascertain from morphological characteristics alone which of them developed into the pulmonary fluke. Consequently,



the eggs of the pulmonary fluke after hatching into miracidia were allowed to come into contact with several species of fresh water mollusks, of which they infected two. But as it was difficult to keep the two species alive in the aquarium long enough to get cercariæ, the second intermediate hosts of the pulmonary distomas were looked for in the severely infected villages of the savage tribes.

3. The miracidia of the pulmonary distomas leave the egg about 4 weeks after they are first set free in the water, and if they do not reach mollusks they soon die.

4. Three species of fresh water mollusks were found to act as the first intermediate host of the pulmonary distomas; viz., *Melania libertina* Gould, *Melania tuberculata* Mueller, and *Melania oblique-granosa* Smith.

5. The cercariæ of the pulmonary distoma may be identified by their small size and a spine in the oral sucker. They develop in the liver of the three species of *Melania* mentioned above.

6. The second intermediate hosts of the pulmonary distoma, detected in the Prefecture of Shinchiku, are the following three species of fresh water crabs: *Potamon (Geothelphusa) obtusipes* Stimpson (native name, red crab), *Potamon (Geothelphusa) dehaanii* White (native name, dung crab), and *Eriocheir japonicus* De Haan (native name, hairy crab). In addition it was discovered that the following two species might act as intermediate hosts: *Sesarma dehaanii* Milne-Edwards and *Potamon (Parathelphusa) sinensis* Milne-Edwards. In Formosa four of the five species are the carriers of the cercariæ.

7. The encysted cercariæ are found in the gills, liver, and muscle, and have an elongated dark excretory vesicle in the middle of their bodies. They resemble the adult flukes.

8. Full grown encysted cercariæ fed to dogs develop into mature pulmonary distomas and begin to lay eggs in about 90 days.

9. In the final host the parasites are taken into the alimentary canal as encysted cercariæ. They liberate themselves from the cysts in the intestine and bore through the jejunum into the abdominal cavity. They then pierce the diaphragm, enter the thoracic cavity, and piercing the pleura reach the lungs. In the parenchyma of the lungs they form cysts and develop into adult forms.

10. The chief causes of pulmonary distomiasis are the eating of raw or insufficiently cooked crabs infected with the cercariæ of *Paragonimus westermanni*, and the drinking of river water containing them.

In conclusion the author wishes to express his indebtedness to Professor Takaki, Chief of the Scientific Research Institute of the Taiwan Government, and others who have given him valuable assistance.

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## EXPLANATION OF PLATES.

## PLATE 22.

Early developmental stages of *Paragonimus westermanni*.

FIG. 1. Eggs in fresh sputum. Zeiss oc. 2, obj.  $\frac{1}{2}$ , oil immersion.

FIGS. 2 to 4. Eggs in various stages of development. Zeiss oc. 2, obj.  $\frac{1}{2}$ , oil immersion.

FIG. 5. Free swimming miracidia. Zeiss oc. 2, obj.  $\frac{1}{2}$ , oil immersion.

FIGS. 6 to 8. Sporocysts in *Melania libertina*. Zeiss oc. 2, obj.  $\frac{1}{2}$ , oil immersion.

FIG. 9. Cercaria developed in *Melania*. Zeiss oc. 2, obj.  $\frac{1}{2}$ , oil immersion.

FIGS. 10 to 14. The youngest cercariæ that lie in the liver of the fresh water crab, *Potamon obtusipes*. Zeiss oc. 2, obj. AA.

FIGS. 15 to 18. Encysted young cercariæ in the liver of a crab. Zeiss oc. 2, obj. AA.

FIG. 19. Full grown encysted cercaria. Zeiss oc. 2, obj. AA.

## PLATE 23.

FIG. 20. Fresh water univalves, the first intermediate host of *Paragonimus westermanni*. Natural size. a, *Melania libertina* Gould; b, *Melania obliquegranosa* Smith.

FIG. 21. Fresh water crab, *Potamon obtusipes* Stimpson, the second intermediate host of *Paragonimus westermanni*. Natural size.

FIG. 22. Fresh water crab, *Potamon dehaanii* White, the second intermediate host of *Paragonimus westermanni*. Natural size.

## PLATE 24.

The second intermediate hosts of *Paragonimus westermanni*.

FIG. 23. *Potamon sinensis* Milne-Edwards. Natural size.

FIG. 24. *Sesarma dehaanii* Milne-Edwards. Natural size.

FIG. 25. *Eriocheir japonicus* De Haan. Natural size.

## PLATE 25.

FIG. 26. Jejunum of a kitten experimentally infected by *Paragonimus westermanni*. Natural size. *a*, petechiæ; *b*, mesentery.

FIG. 27. Omentum of the same film to show the worm attached on the surface. Natural size. *a*, young worm; *b*, stomach.

## PLATE 26.

FIG. 28. Diaphragm of a kitten that had been experimentally infected by *Paragonimus westermanni*. Natural size. *a*, young worm just piercing through the muscular layer; *b*, petechiæ produced by the worm; *c*, tendinous region of the diaphragm.

FIG. 29. Liver of the animal shown in Fig. 28. Natural size. The brown irregular lines indicate the path of the young worm.

## PLATE 27.

FIG. 30. Intercostal muscle of a kitten that had been experimentally infected by *Paragonimus westermanni*. Natural size. *a*, ribs; *b*, intercostal muscle; *c*, young worm.

FIG. 31. Lungs of a pup experimentally infected by *Paragonimus westermanni*. The specimen was examined a few days after feeding. Natural size. *a*, petechiæ.

FIG. 32. Lungs of a pup experimentally infected by *Paragonimus westermanni*, 50 days after feeding. Several cysts are present. Natural size. *a*, cysts.

## PLATE 28.

FIG. 33. Section of the liver of *Melania libertina* that harbors the cercariæ. *a*, liver parenchyma; *b*, cercariæ. Zeiss oc. 2, obj. DD.

FIG. 34. Section of the gills of *Potamon obtusipes*, infected by encysted cercariæ. *a*, encysted cercariæ. Zeiss oc. 2, obj. AA.

## PLATE 29.

FIG. 35. Section of the jejunum of a kitten experimentally infected. *a*, mucous membrane; *b*, connective tissue; *c*, internal muscular layer; *d*, external muscular layer; *e*, young distoma piercing through the intestinal wall; *f*, worm track. Zeiss oc. 2, obj. AA.

FIG. 36. Section of the diaphragm of a kitten experimentally infected. *a*, young worm in the muscle of the diaphragm. Zeiss oc. 2, obj. AA.

## PLATE 30.

FIG. 37. Section of the liver of a kitten experimentally infected, showing the hemorrhagic spots caused by the young worm. *a*, hemorrhagic spots. Zeiss oc. 2, obj. AA.

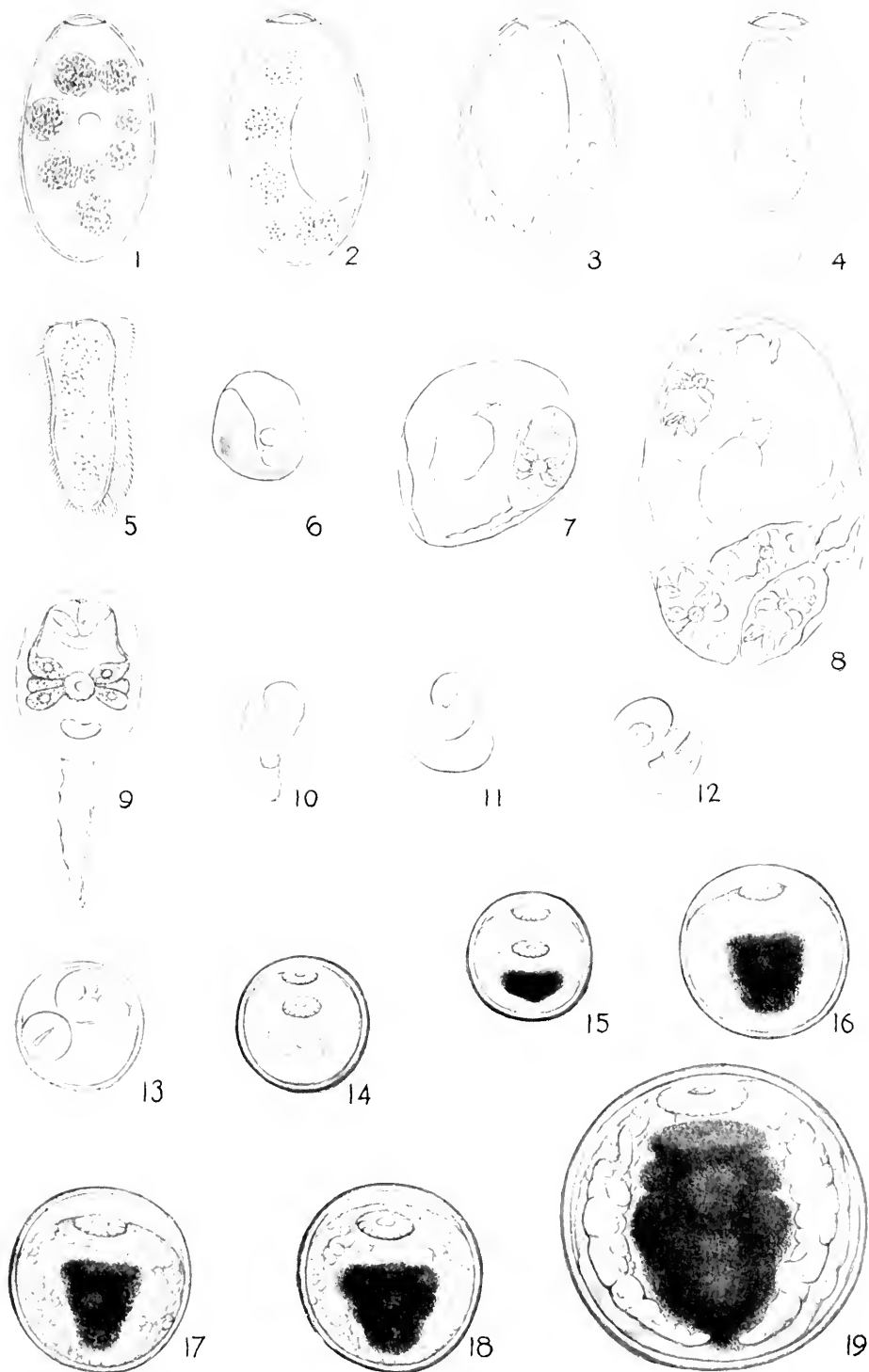
FIG. 38. Section of the lung of a pup experimentally infected; 60 days after feeding. *a*, half grown worm. Zeiss oc. 2, obj. AA.

## PLATE 31.

Various developmental stages of *Paragonimus westermani* in the final host.

- FIG. 39. Young worm, 3 days after feeding.  $\times 30$ .  
FIG. 40. Young worm, 14 days after feeding.  $\times 30$ .  
FIG. 41. Young worm, 21 days after feeding.  $\times 30$ .  
FIG. 42. Young worm, 25 days after feeding.  $\times 30$ .  
FIG. 43. Half grown worm, 50 days after feeding.  $\times 15$ .  
FIG. 44. Mature worm 90 days after feeding.  $\times 15$ .

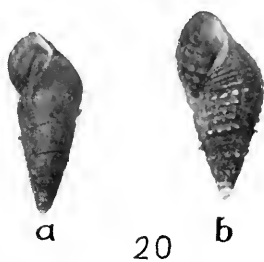




(Nakagawa: Pulmonary distomiasis.)







(Nakagawa; Pulmonary distomiasis.)





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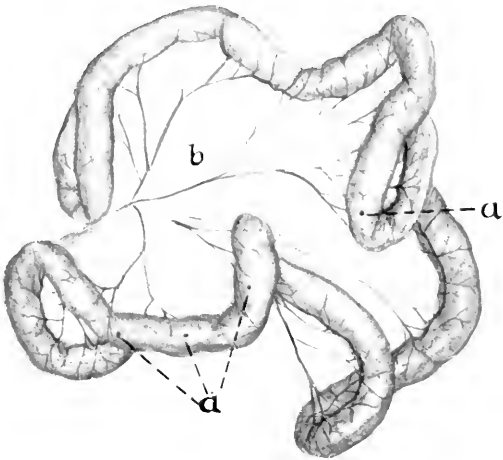


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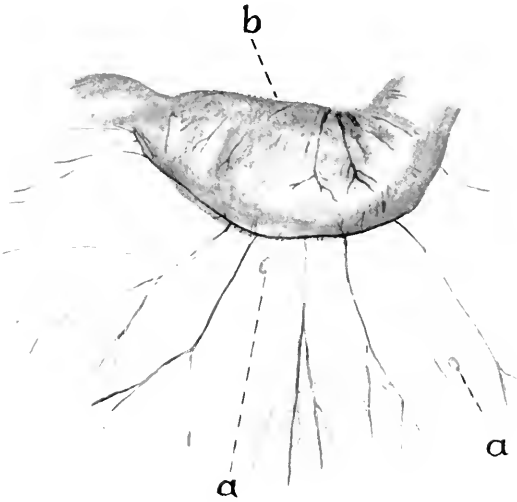


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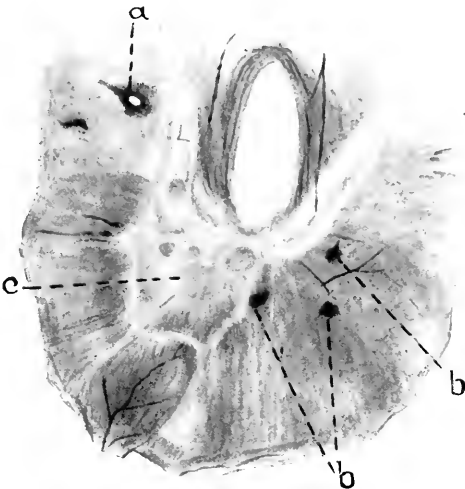


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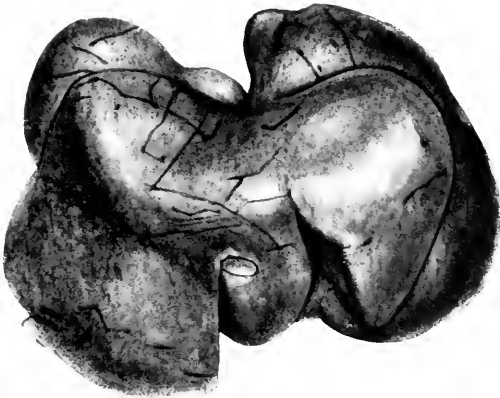


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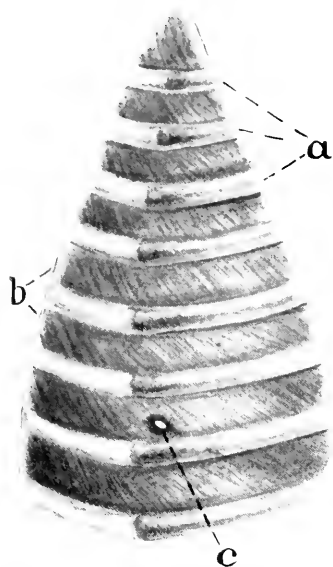
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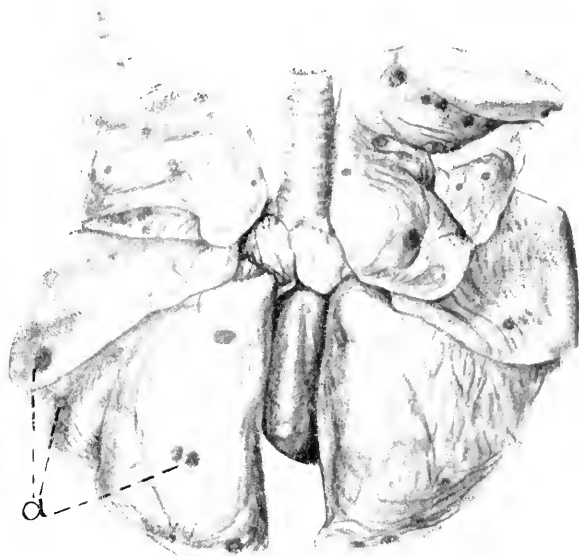
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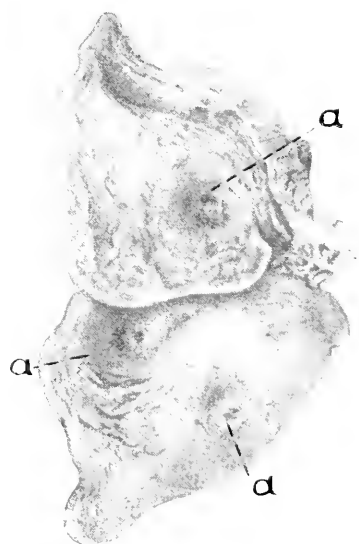




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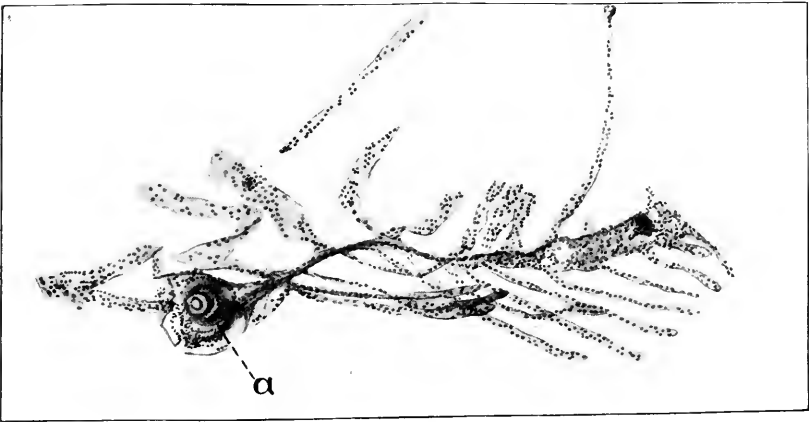
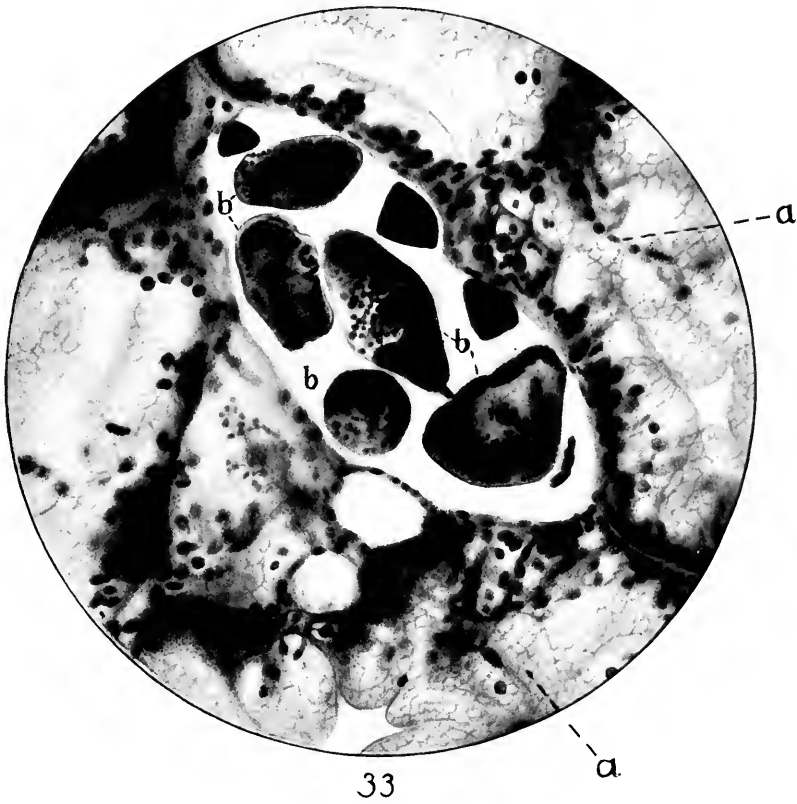
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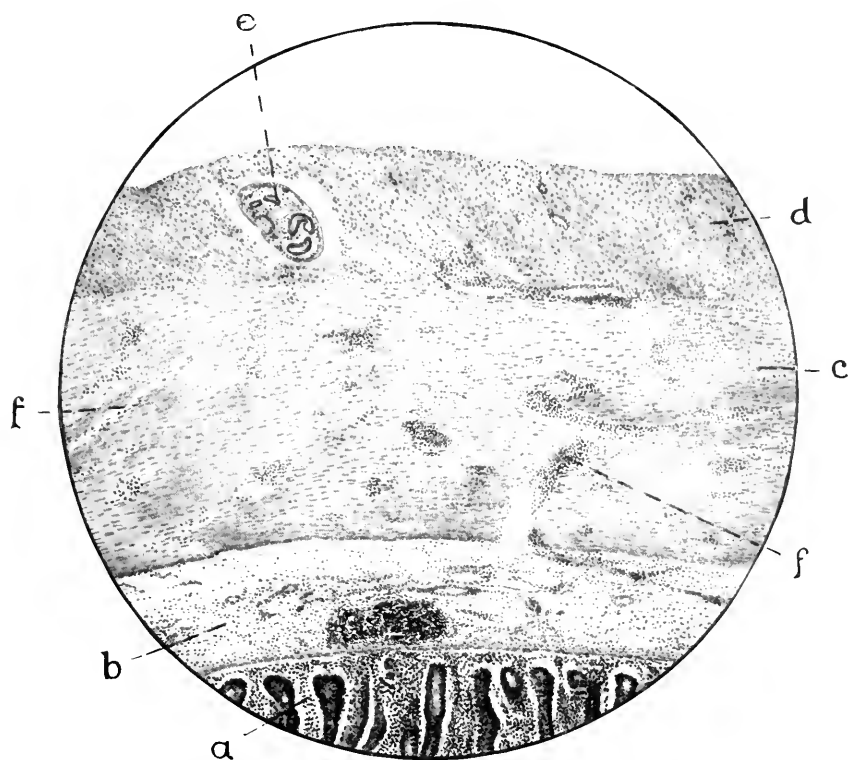
(Nakagawa: Pulmonary distomiasis.)





(Nakagawa: Pulmonary distomiasis.)



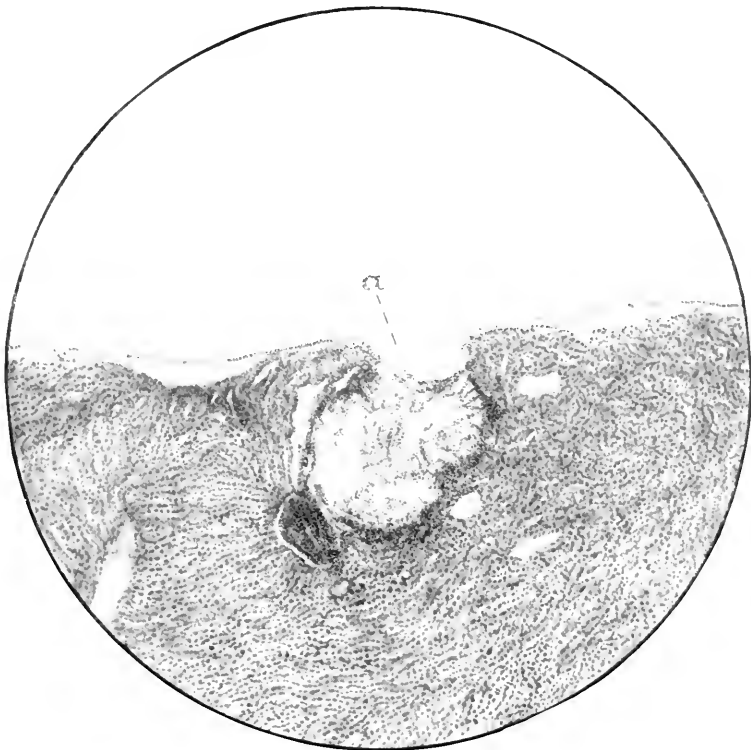


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a

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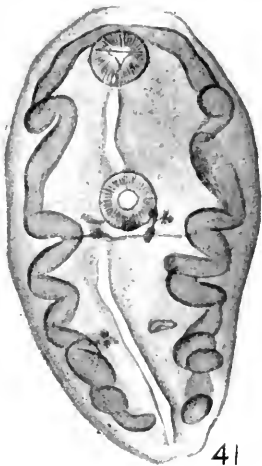




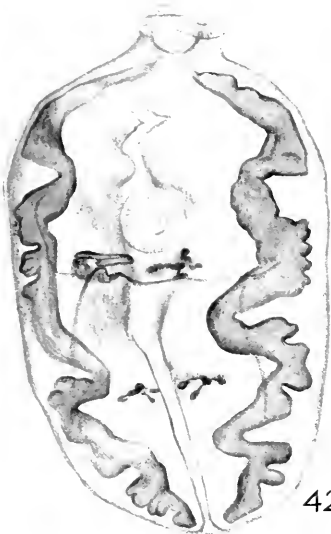
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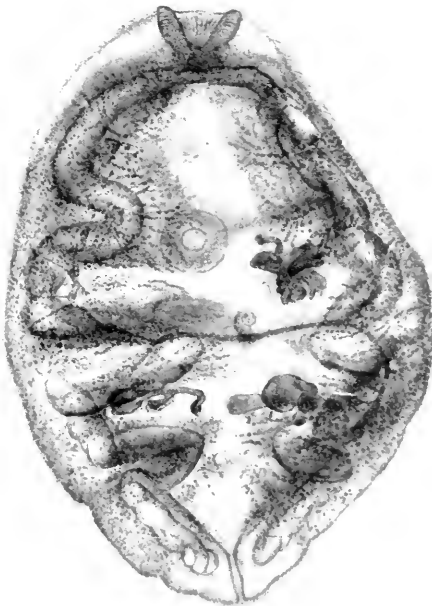
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(Nakagawa: Pulmonary distomiasis.)



## THE DISTRIBUTION IN THE HUMAN BODY OF SPIROCHÆTA ICTEROHÆMORRHAGIÆ.\*

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The material examined came from autopsies performed on forty-three patients who died at various stages of illness, as shown in the table. The ages ranged from 2 to 73 years. There were 36 males and 7 females. 5 patients had cirrhosis of the liver, 9 a mixed infection. In 19 cases, serum was administered in varying quantities; 1 was treated with salvarsan. In a number of cases, only the vital organs were examined. As a rule, these organs were placed in a 10 per cent formalin solution; a few were treated with Orth's fluid. All the material examined was impregnated with silver, according to Levaditi's method.

### *General Distribution of the Spirochetes.*

Inada<sup>1</sup> and Ido have divided the progress of Weil's disease into three clinical stages; (1) febrile, (2) icteric, (3) convalescent. As the distribution of the spirochete varies in the different stages, which are not sharply demarcated, we shall discuss the findings for each stage separately.

*Febrile Stage.*—This period continues to the 6th or 7th day of illness. The clinical symptoms are fever, intestinal disturbances, headache, muscular pains, hyperemia of the conjunctiva, and albuminuria. The blood is pathogenic for guinea pigs. No immune bodies could be demonstrated by Pfeiffer's test.

Spirochetes in large numbers are present. Their distribution resembles in general that in the experimental guinea pigs. The organisms are lodged mainly

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\* Presented in April, 1916, before the Japanese Congress on Internal Medicine and the Japanese Pathological Congress.

<sup>1</sup> Inada, R., The clinical aspects of spirochætosis icterohæmorrhagica or Weil's disease, *J. Exp. Med.*, 1917, xxvi, 355.

in the liver, kidneys, and suprarenals. Immune bodies are present as early as the 5th day, and we may assume that they exist even prior to that time, but as death does not usually occur so early in the disease, opportunity was lacking for substantiating this point. In the patient treated with salvarsan, who died on the 6th day,<sup>2</sup> spirochetes were found in the liver. That organ showed cirrhotic changes, and it is possible that the irregular distribution is related to this condition, although a similar distribution was found in a patient dying on the 8th day, as well as in the animals treated with serum or salvarsan. We believe that the destruction of spirochetes in the liver and their irregular distribution are due to the presence of immune bodies. The spirochetes in the suprarenals of this patient were distributed in groups.

By the 7th day the spirochetes in the liver have been almost completely destroyed, and are found but rarely thereafter.<sup>3</sup> We found only a few in 2 cases on the 7th day, in 5 cases on the 8th day, and in 8 cases on the 9th day of illness.

In the suprarenals spirochetes have not been clearly demonstrated on the 7th day, but it is certain that by the 8th day their almost complete destruction has been attained. The organisms are usually found in the kidneys on the 6th and 7th days.

Numerous spirochetes were also found in the pancreas, cardiac muscles, in the intestinal wall, the prostate, testicles, epididymis, the walls of the urinary bladder, and the arteries. The spleen and the lymph glands showed, also in the experimental animals, a small proportion of spirochetes.

The distribution of *Spirochæta icterohæmorrhagiæ* in human beings on the whole parallels that found in guinea pigs, with the exception that in man the organisms are more loosely and irregularly scattered and also show a greater degree of degeneration. These differences are attributable to the immune bodies. It is evident from the infection experiments made with the blood of patients that development of immune bodies is already under way as early as the 5th or the 6th day. No opportunity has presented itself to investigate the distribution of spirochetes at the beginning of illness, before the immune bodies have appeared, but recently we observed a patient who on the 4th day showed numerous spirochetes in the blood, similar to the findings in the guinea pig.

<sup>2</sup> This case was kindly put at our disposal by Dr. Takeya, Chief of the Second Medical Clinic, to whom we are greatly indebted.

<sup>3</sup> Dr. Iida, Assistant in the Second Medical Clinic, in one case found an abundance of spirochetes in the liver of a patient dying on the 8th day.

*Icteric Stage.*—This stage covers a period from the 7th or 8th to the 12th or 13th day of illness. The icteric condition is then at its height and the mortality is greatest. The proportion of autopsies was twenty-one out of forty-three cases. Further development of the immune bodies takes place during this stage, and they can be demonstrated in the blood.

In the course of this period the spirochetes disappear from the blood, and infection experiments are usually negative. Destruction of the spirochetes in the organs takes place. They disappear almost completely from the liver and the suprarenals, leaving only a slight degenerated residue,<sup>4</sup> but can be traced more or less readily in the kidneys, cardiac muscles, skeletal muscles, particularly the gastrocnemius, and rectus abdominis, the walls of the intestine, especially the appendix, large intestine, and stomach, prostate, urinary bladder, testicles, and epididymis, thymus, and uterine muscles. They are most constantly found in the kidneys and the cardiac muscles, but even here the organisms are not very numerous.

On the whole, the spirochetes during this stage are more abundant in the cells (epithelial, muscular, etc.). Those located in the kidneys are found in the tubules. There are massed foci in the interstitial spaces. Frequently the spirochetes are found in coagulated, homogeneous, or hyaline substance, such as urinary casts, degenerated hyaline muscle cells, etc.

*Convalescent Stage.*—This stage begins variously from the 13th to the 16th day of illness. The immune bodies are then fully developed, and spirochetes are abundantly excreted with the urine.

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<sup>4</sup> The abundance of spirochetes in Dr. Iida's case (8th day) must be considered exceptional.

In the table the following signs are used:

\* Cirrhosis of liver.

† Cirrhosis and cancer of liver.

‡ Mixed infection.

+, very sparse distribution of spirochetes; 1 or a few to a specimen. +, sparse distribution. Spirochetes can be found readily; 10 to 20 to a preparation, 1 in one or more fields. ++, spirochetes relatively numerous, 1 or more to a field, many in a preparation. +++, spirochetes numerous; some in every field; perhaps many in a single field. —, negative. No spirochetes in 1 or more specimens.

The immune serum was given chiefly intravenously; the figures in bold-faced type indicate subcutaneous administration.

Case 12 was placed at our disposal through the kindness of Dr. Iida, Assistant in the Second Medical Clinic of the University; Case 41 by Mr. Onuma, Director of the Chibaken Board of Health. We desire herewith to express our thanks.

No.	Age.	Sex	Day of illness.	Hours until autopsy.	Treatment.	Fixing fluid.	Liver.		Kidney.		Suprenals.	Spleen.	Lymph glands.	Intestines.	Tongue.	Salivary gland.	Pancreas.	Lung.	Trachea.	Thyroid.	Thymus.	Heart.	Arteries.	Muscles.	Skin.	Nervous system.	Urinary bladder.	Testicles.	Prostate.	Uterus and ovary.		
							Extracellular.	Intracellular.	Interstices.	Tubules.																						
1	30	M.	523	20 cc. serum 5th day.	Formalin.		+		+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
2*	59	"	618	20 cc. serum 4th and 5th days.			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
3	57	"	64	Salvarsan.			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
4†	48	"	6				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
5*	45	"	725				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	47	"	7				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	28	"	822				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	44	F.	83	60 cc. serum 4th day; 20 cc. 5th day.			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	34	M.	820				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
10	49	"	8				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	56	F.	812	20 cc. serum 6th and 7th days.			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12		M.	8				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
13	59	"	917	40 cc. serum 6th and 7th days.			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	22	"	916		Orth's fluid.		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
15†	70	F.	919				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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In the organs, with the exception of the kidneys and the heart, complete destruction of spirochetes has taken place. The kidneys, on the other hand, show the organisms constantly, and particularly in the tubules. In Case 42 they could be demonstrated in the urine up to the 55th day. The experimental animals treated with immune serum and salvarsan also harbored spirochetes in their kidneys for a long time after recovery. It was observed in white rats, mice, rabbits, and occasionally in guinea pigs, that the spirochetes lodge solely in the kidneys.<sup>5</sup> The relationship of the spirochetes to the kidneys is noteworthy in that the immune bodies are ineffectual against the organisms contained in the kidney tubules. The spirochetes found occasionally in the cardiac muscles are mostly degenerated.

Summarized briefly, the distribution of the spirochetes in the various organs of the human body differs according to the degree of development of the immune bodies. The spirochetes disappear first from the liver and suprarenals, but remain for some time in the muscles, prostate, thymus, appendix, testicles, epididymis, etc. In the kidneys they can be found for a long time. Their mode of disappearance would seem to indicate distribution varying with the different stages of the disease. And not alone the general distribution, but the local position of the spirochetes changes with the different stages. In the early stage, they are located mainly extracellularly, in the interstices; in the later stage, owing to the development of the immune bodies, in the blood, in the main intracellularly, and within the kidney tubules.

#### *Distribution of Spirochæta icterohæmorrhagiæ in the Tissues.*

In the first stage of Weil's disease the organisms are located extracellularly, in the tissues.

Spirochetes are rarely found in the epithelial cells, the kidney tubules, and other glands. They are lodged chiefly in the interstitial spaces surrounding the cells, frequently around the periphery of cells. This position is characteristic, and typical in guinea pigs. In man, in whom a development of immune bodies

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<sup>5</sup> Drs. Ido, Hoki, Ito, and Wani, after careful examination of the various organs of house rats were able to find *Spirochæta icterohæmorrhagiæ* only in the kidneys. Ido, Y., Hoki, R., Ito, H., and Wani, H., The rat as a carrier of *Spirochæta icterohæmorrhagiæ*, the causative agent of Weil's disease (spirochætosus icterohæmorrhagica), *J. Exp. Med.*, 1917, xxvi, 341.

takes place in the blood, the distribution is somewhat different. Here the spirochetes are more frequently enclosed in the cells and cell tubes, and increasingly so with the progress of the disease. The spirochetes disappear first from the interstitial spaces, remaining for some time longer in the cells and kidney tubules. This phenomenon is less marked in the liver and suprarenals, where the spirochetes are destroyed at an early stage; it is very marked in the kidneys. Spirochetes proliferate in the kidney tubules and are excreted with the urine.

Muscular tissue, such as that of the heart and skeletal muscles, retains the spirochetes for a relatively long time. The organisms are seen to surround the muscle fibers closely, but are rarely found within them, though this may be the case in late stages of the disease. In a patient dying on the 9th day, we observed densely massed spirochetes in degenerated hyaline muscle fibers. It seems as though the spirochetes were able to penetrate more readily degenerated or broken cells than those that are intact. This is also true of epithelial cells. Smooth muscle reacts in the same manner as striated. The muscular tissue of the prostate, urinary bladder, gall bladder, the vessel walls, and the intestinal walls are lodging places during the second stage. On one occasion we observed numerous spirochetes in the medullary substance of the suprarenals, while the cortex was already free from spirochetes. In smooth muscle, the organisms are not located parallel to the muscle fibers, but cross them irregularly. The spirochetes occur but rarely in the interstices of the nervous system.

The connective tissue contains spirochetes in abundance. It is, in fact, a main repository for them. Carried by the blood stream, they reach first the walls of the capillaries, and from there are spread over the surrounding connective tissue. Hence they are to be found in great numbers in the lumen, the walls, and the perivascular connective tissue of the capillaries. In man by the 6th day the spirochetes have left the capillaries, having proceeded by way of the delicate fibrous strands, deep into the parenchyma. The coarse connective tissue shows a sparse distribution. The spirochetes are assembled mostly in the dividing tissue lying between the connective tissue and the parenchyma, or in the narrow interstitial layer of connective tissue. They disappear relatively early from the connective tissue, though scattered specimens may be observed in the coarse connective tissue for a long time. The spirochetes may also be carried by the lymph channels, though it is difficult to demonstrate them in the lymphatic vessels.

The spirochetes are often harbored for some time in homogeneous substances, such as urinary casts, degenerated muscle fiber, in colloidal masses of the thyroid, and other coagulated masses. They seem to show a preference for substances of this kind, where they can be secure against the action of the immune bodies.

*Phagocytosis.*—In the early stage spirochetes are rarely found enclosed in leukocytes, endothelial cells, or connective tissue cells; but in the course of the

disease they are increasingly found in phagocytes. This observation was confirmed experimentally. Phagocytosed spirochetes were found in great numbers following the injection of immune serum into guinea pigs. In man, the phenomenon is not quite so marked, owing perhaps to the fact that the development of the immune bodies is a gradual process, while in the animal large numbers of immune bodies are introduced suddenly. The spirochetes contained in phagocytes are in various stages of degeneration. It would appear as if the spirochetes first degenerate and are then taken up by the phagocytes. Organisms not degenerated but showing regressive changes are sometimes found in phagocytes at the beginning of illness. From this we may assume that they actively penetrate the degenerated phagocytes, and are passively received by the latter.

### *Distribution of Spirochetes in the Organs.*

*Liver and Bile Ducts.*—In our experimental work with guinea pigs we found that the liver contained the densest distribution of spirochetes, while in man the organisms are rather scattered in that organ. Only in one patient dying on the 6th day and in one of Iida's 8 day cases were we able to demonstrate them numerously. In the densest region, ten to twenty were counted to the optical field. As a rule, the organisms are located extracellularly. Within the lobes they are found close to the fine fibrous strands or between the hepatic cells. In our cases they were associated intimately with markedly developed fibrous strands in the periphery of the acini. In the hepatic cells and the stellated cells of Kupffer they are found but rarely, and then in degenerated condition. The spirochetes located between the hepatic cells are bent and adhere with their ends or bent sides to the cells. At times they are located in almost parallel lines on the periphery, giving the appearance of lying in the intercellular bile duct capillaries, but their demonstration within the capillaries is difficult. Only in one instance were we able to observe a spirochete in a somewhat dilated biliary duct. It is not probable that the spirochetes stand in any close relation to the bile duct capillaries. In man they are also not so closely related to the blood capillaries as in the guinea pigs. They are rarely present in the interstices of the lobes; *i.e.*, in the interlobular connective tissue. They are infrequently found massed about the acini and the bile ducts; they enter often into the wall epithelium of the ducts, but almost never into the lumen. They are also rare in the zone of leukocytic infiltration, as well as in the interstitial blood vessels, and are seldom found in the lumen. The spirochetes in the liver are frequently contained in degenerated cell masses and coagulated homogeneous substances. On the whole, distribution within the liver is irregular. In the course of the disease, the spirochetes in the liver are almost completely destroyed, and those found occasionally enclosed in stellated cells are mostly of the degenerated type.

The large bile ducts and the gall bladder were examined in five patients who died during the second stage of Weil's disease. Very rarely were spirochetes found in the subepithelial fibromuscular layer.

*Kidneys.*—The kidney is characterized by a more or less dense distribution of spirochetes throughout the course of the disease. We were able to demonstrate them up to the 55th day of illness. In cases of mixed infection treated with immune serum, scattered specimens are found in the kidneys after they have disappeared from other organs. In fact the organisms could be found in varying numbers in the kidneys of all cases.

In earlier stages the spirochetes are present mostly in the interstices, more numerous in the cortex than in the medullary substance. They are found in the fibrous strands (trabeculæ), in the tissue spaces, and in the interstitial cells. They are intimately related to the capillaries. In the kidney tubules they are found surrounding the tunica propria. They are rarely contained in the epithelium and the lumen of the kidney tubules. In the course of the disease part of the spirochetes are gradually destroyed, but even in later stages, numerous organisms can be detected in the tubules. In the convalescent stage, innumerable, thickly grouped spirochetes are found in the tubules. It is evident, therefore, that the spirochetes proliferate in the tubules. The number found in the kidneys does not parallel the course of illness. In a patient dying on the 8th day without complicating symptoms, the organisms were sparse, while in one dying on the 10th day, spirochetes in large numbers were found. The latter case was of interest because the spirochetes were thickly grouped in the interstices. In the densest regions, the organisms covered the whole area of the interstitial space and closely surrounded the tubules. It was possible to observe them by low power of the microscope as black dots or spots. Occasionally one sees a case in which the organisms are found mainly in the tubules.

The spirochetes in the tubules lie in the pockets of the lumen, close to the epithelium. They are to be found in the detritus mass or in urinary casts, seldom in epithelium, sometimes in vascular desquamated epithelium.

The spirochetes in the tubules are frequently located in the border zone between the cortex and the medullary substance, the free organisms in the convoluted tubules, and those enclosed in casts in the straight tubules.

In man the spirochetes were never observed in the glomeruli, though in guinea pigs they were found there in sparse numbers. We may conclude, therefore, that the spirochetes in the tubules are not flooded out by means of the glomeruli, but rather that those contained in the interstices migrate through the walls of the tubules directly into the lumen, where they are preserved and may proliferate.

*Suprarenals.*—The suprarenals, like the kidneys, are characterized in the guinea pig by a dense distribution of spirochetes. Many were seen in a patient dying on the 6th day. They were contained in the interstices, combined with the interlobular fibrillæ of the upper cortex layer. In the cortex cells and their sheaths, they were scarce. They were irregularly distributed, and were found mainly in the subcapsular layer, almost never in the medullary, and the deep cortex layer. After the 8th day, only a few degenerated residual forms are present. The latter lodge in the cortical layer and in the interstitial cells. Occa-

sionally spirochetes are located extracellularly, between the cortical cells and in the interstices. In a patient dying on the 8th day we observed a number of intact specimens in the medullary muscles.

*Spleen, Lymph Glands, and Bone Marrow.*—The hematopoietic organs showed throughout a sparse distribution of spirochetes. In the spleen the organisms are found at an early stage in the pulp, trabeculae, and capsular tissue, very rarely in the Malpighian bodies. In the experimental animals it was also difficult to find spirochetes in the Malpighian bodies, particularly in the center. In the pulp tissue, the spirochetes are found mainly in the lattice fibers and the fibrous strands of the sinus and vessel walls. They are likewise present in the pulp cells and in the phagocytes. The phagocytized spirochetes are mostly degenerated. On the other hand, the spirochetes found in the trabeculae and other coarse fibrous tissue are relatively well preserved. Spirochetes are sometimes found in the walls of large arteries and in their vicinity.

Further on in the disease, spirochetes are rarely present in the trabeculae, in coagulated masses, and in phagocytes. They are then mostly degenerated in type, though sometimes in good condition in the trabeculae.

Numerous organisms are contained in swollen lymph glands at the beginning of illness. At first, they are present in the regions of the lymph vessels and sinuses. On the 2nd and 3rd day of illness they appear deeply seated in the fibrous tissue of the parenchyma. The vessel walls, the trabeculae, and the perivascular tissue also show them. Spirochetes are found extracellularly in the peripheral zones of the follicles. Phagocytized spirochetes are rare. On the 3rd and 4th day we find them numerous in the lumen of the vessels. It would seem, therefore, that the organisms gain access to the lymph channels through the local lymph glands. From there they go into the parenchyma and blood vessels. This phenomenon was confirmed on animals. After the 7th day, it is difficult to find spirochetes even in swollen lymph glands. In the mesenteric glands, they are more numerous, but degenerated and contained mostly in phagocytes. They are rare in fibrous strands. Later on, only scattered residual forms are seen in phagocytes and fibrous tissue.

The conditions in the lymphoid tissue of the various organs and the tonsils resemble those of the lymph glands.

The bone marrow, particularly that of the tibia, was studied in a few cases. In a patient dying in the second stage, no spirochetes were found. In guinea pigs, on the other hand, spirochetes were as numerous in the bone marrow as in the blood.

*Digestive Tract and Pancreas.*—In guinea pigs closely packed spirochetes are found in the subepithelial mucosa of the intestinal wall. In man their distribution in the intestinal wall was rather scanty on the 7th day and throughout the second stage. In the appendix wall and that of the large intestine and stomach, the spirochetes are more numerous at this stage. They are scattered throughout the mucosa, somewhat more numerous in the submucosa and muscular layer.

They are found free in the tunica propria or in phagocytes, and are often lodged close to the epithelial layer, rarely in the layer itself. They are present for a long time in the muscular layer, and often in considerable numbers. In the convalescent stage, spirochetes do not occur in the intestinal wall; but occasionally a few are found in the intestinal crypts and lumen, though we are unable to say definitely that the organisms were *Spirochæta icterohæmorrhagiæ*, as they were very closely grouped and well formed, which are not characteristics of this spirochete. Moreover, in the silver preparation they did not appear granular, and similar organisms are often found in healthy persons. Dark-field illumination is required to decide this point.

Judging by analogy with the kidneys, it may be supposed that the *Spirochæta icterohæmorrhagiæ* wander out into the intestinal lumen. Infection experiments carried out on guinea pigs with intestinal contents were occasionally positive. In some cases, the organisms may be identified with certainty in the intestinal lumen. These spirochetes are clearly granulated and are located in desquamated epithelium and detritus masses.

The esophagus, tongue, and salivary glands also harbor the spirochetes in varying numbers. As a rule, they lodge in the interstices early in the disease, and later on in the cells.

The pancreas showed spirochetes on the 6th or 7th day. In the densest region a number of specimens appeared in the optical field. They occur mainly in the interstices, closely surrounding the body of the gland and the excretory duct, rarely in the lumen and in the epithelial cells. Later in the disease only a few degenerated forms were found, mostly in the cells.

*Respiratory Organs, Thyroid, and Thymus.*—The lungs contain but few spirochetes, only a small number being found in the alveoli on the 6th day. They were enclosed in desquamated epithelium, blood, and blood coagulum. Later on, it is almost impossible to find them; very rarely one finds degenerated forms in the alveolar epithelium and coagulated masses.

In the trachea spirochetes were present in the interstices on the 7th day in considerable numbers. In another individual, dying on the 8th day, they were scattered.

A few spirochetes were present in the interstices and capsule of the thyroid in a patient who died on the 6th day. At a later stage they are rarely found in the colloidal masses

The thymus, which was examined in two cases, showed a relatively thick distribution of spirochetes. In a patient dying on the 10th day, they were found rather plentifully in the capsule and septum tissue, and also enclosed in thymus cells. The other patient, who had died on the 12th day, had received serum treatment. Here also numerous organisms were present, mainly degenerated forms, located extracellularly.

*Circulatory System.*—In the search for spirochetes in man, particular attention has been paid to the heart, for the number there is much greater than in guinea

pigs. On the 7th day of illness and throughout the second stage, spirochetes are more or less numerous; a few were seen in an optical field. Early in the disease they are found in the epicardium and endocardium, and most constantly in the muscular layer. They occur first in the interstices, later on in muscle cells. In the interstices they are found in the perimysium, closely surrounding the muscle cells, in the neighborhood of the capillaries, in coagulated venous blood, and in phagocytes. Those enclosed in cells are found in the periphery and in the centers of muscle cells, independent of the striation. On the whole, they are more numerous in the right side of the heart than the left. The sinoauricular region also showed a dense distribution. During convalescence spirochetes are found here and there, mostly degenerated.

In the arterial walls of the aorta, carotids, brachials, and radials, spirochetes are present more or less constantly during the second stage, chiefly in the media, seldom in the adventitia. They are not numerous. In the lumen of the brachial artery, we observed a single specimen in a patient dying on the 9th day. This was located in coagulated blood near the wall.

*Skeletal Muscles and Skin.*—The skeletal muscles showed on the 7th day (second stage) a relatively dense distribution, particularly in the calf muscle and rectus abdominis, where the histological changes were most marked. On the 9th day a few specimens were seen. The spirochetes found in muscles occur as a rule in the interstices, *i.e.*, the perimysium, and they surround the muscle fibers closely. Only once did we find thickly grouped spirochetes in degenerated hyaline muscle fibers. At a later stage the organisms are rarely seen in muscle fiber.

The outer skin rarely shows spirochetes. We could discover them only in two out of four cases—in the one contained in prickly cells, in the other in the corium. None were found in the lumen of the sweat and sebaceous glands.

*Nervous System.*—At an early stage (the 6th day) spirochetes were found in the interstices and the spinal meninges. In rare instances they were present in the interstices of the central and peripheral nervous systems. In the gray and white substance of the brain and spinal cord, degenerated forms were found on a few occasions. Once they were present in the neuroglia.

*Urinary Bladder and Genital Organs.*—On the 7th day, and during the second stage, a large number of spirochetes were present, particularly in the muscular layer of the bladder. In the testicles and epididymis, well preserved specimens were seen on the 6th and 7th days (second stage), in the fibromuscular tissue, but seldom in the tubules. The prostate shows an unusual condition. On the 7th day (second stage), numerous spirochetes were found in the fibromuscular tissue. The muscular layer of the uterus and the fibrous tissue of the ovaries may show scattered spirochetes, those in the uterus being in well preserved condition.

*Factors Influencing the Distribution of Spirochæta icterohæmorrhagiæ.*

*Severity of Illness.*—Those cases of Weil's disease, in which the pathological changes are particularly marked, show in the main the densest distribution in the various organs. In guinea pigs also the distribution of spirochetes is more or less paralleled by the degree of icterus and hemorrhage.

*Mixed Infections and Complications.*—Suppurative or septic infections, which often accompany Weil's disease, greatly influence the distribution; the spirochetes in the organs thus become reduced in number. They are often absent from all the organs, except the kidneys and the cardiac muscles. Cirrhosis of the liver does not seem to affect the distribution.

*Serum and Salvarsan Treatments.*—The spirocheticidal action of immune serum has been previously described.<sup>6</sup> The statements then made are supported by our postmortem findings. Very few spirochetes are discovered in the organs, with the exception of the kidneys, where they remain for a long time. The spirochetes in other organs had evidently been destroyed.

*Types of Spirochete in Human Tissues.*

In comparison with those found in the experimental guinea pigs the spirochetes in man are irregular in type. They show greater rigidity than those in the animals. They are atrophic and shrunken, and of varying thickness. The edges are not smooth and the curves are irregular; many small waves are observed. At one or both ends, or in the middle, a circumscribed thickening may be present. Sometimes one-half of the spirochete differs in thickness from the other. The circumscribed thickening can be distinguished from the bud, as the latter is more markedly granular. The spirochetes may appear as large, irregularly distributed chains of granules. One end often shows an enlarged, granular bud. This rare form was distributed in a number of organs. It seems to arise through a loosening and

<sup>6</sup> Inada, R., Ido, Y., Hoki, R., Ito, H., and Wani, H., The serum treatment of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 485.



enlargement of some of the granules of the spirochetes. Abortive forms (rod-like, comma-shaped, or bent) arise through the breaking off of parts of the spirochete. One end may give the appearance of having dissolved. The large bud may be present at one end of a markedly shrunken spirochete. Such deformed types are often found in phagocytes.

In the group, where the spirochetes are closely assembled, many small granules or rings are seen; these stain black when impregnated with silver. The granules are globoid or irregularly formed, and are from two to five times as thick as the spirochetes. They are detached, fused buds or broken parts of spirochetes. The rings referred to above are round or somewhat irregular, also from two to five times as large as the thickness of the spirochete. Their origin is identical with that of the granules. On the other hand, the ring type of spirochete which has already been described, is large and irregular, and can be readily distinguished from the rings referred to above.

We are led to believe that these forms are degenerated products, caused by the action of the immune bodies. This point requires further investigation.

In closing we desire to express our thanks to the Director, Dr. Ryokichi Inada, for his assistance in the work, and to Dr. H. Nakayama of the Pathological Institute of Kyushu University for placing at our disposal material for investigation.



THE RAT AS A CARRIER OF SPIROCHÆTA ICTERO-  
HÆMORRHAGIÆ, THE CAUSATIVE AGENT OF  
WEIL'S DISEASE (SPIROCHÆTOSIS  
ICTEROHÆMORRHAGICA).\*

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In a previous communication<sup>1</sup> on the prophylaxis of spirochætosis icterohæmorrhagica, we discussed briefly the excretion and means of invasion of the causative agent. It was clear that soil and water, particularly stagnant water, are related to the infection, but where the spirochetes have their habitation outside of the human body and proliferate until they again attack man remained a difficult problem of prophylaxis.

When Inada at the annual meeting of the Kitasato Institute for Infectious Diseases, in the spring of 1915, gave a comprehensive report of studies made on spirochætosis icterohæmorrhagica in his clinic, Miyajima called attention to the fact that in his investigations on tsutsugamushi, he had found on several occasions spirochetes resembling *Spirochæta icterohæmorrhagiæ* in the kidneys of the field mouse, *Microtus montebelloi*. On the basis of these findings, we conducted during the following year an investigation on twenty-two house and roof rats, *Epymis alexandrinus* and *Epymis norvegicus*. We discovered on one occasion in the kidney of one of the animals a specimen of *Spirochæta icterohæmorrhagiæ*. There was at that time doubt in our minds whether the organism in question had actually resided in the kidney or was a contamination introduced from the outside,

\* Published in *Tokyo Iji-Shuko*, July, 1916, Nos. 1978, 1979.

<sup>1</sup> Ido, Y., Hoki, R., Ito, H., and Wani, H., The prophylaxis of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 471.

inasmuch as we were working with *Spirochæta icterohæmorrhagiæ*. The problem was then left for future investigation.

In the spring of 1916, Miyajima again reported that he had found in *Microtus montebelloi* spirochetes resembling *Spirochæta icterohæmorrhagiæ*, which injected into guinea pigs produced fever and hemorrhage, and after a number of generations, icterus.<sup>2</sup> As the immune serum of *Spirochæta icterohæmorrhagiæ* was capable of destroying the organisms in question, he concluded that they were identical with *Spirochæta icterohæmorrhagiæ*.

From our clinical experience we had already surmised the relation of the rat to the infection in man. Cooks working in kitchens frequented by rats often became ill with spirochætosis icterohæmorrhagica. At the beginning of the year we observed two typical cases following the bite of rats. We were led to the conclusion that the rat plays an important part in the transmission of the infection, and that the spirochete previously found by us in the kidney of a rat was not a contamination, but came from that organ. On the basis of this assumption we undertook an investigation of the house and roof rats in the city of Fukuoka and its vicinity.

#### EXPERIMENTAL.

The rats examined were *Mus alexandrinus* and *Mus decumanus*. We were able to find virulent *Spirochæta icterohæmorrhagiæ* in the kidneys, in 40.2 per cent out of 149 *Mus decumanus*, and in 0.8 per cent of 24 *Mus alexandrinus*.<sup>3</sup> The morphological examinations and

<sup>2</sup> The findings of the Japanese workers have been confirmed in Europe by Dr. Stokes, who conducted an investigation of cases of Weil's disease occurring in the British army. He was able to demonstrate *Spirochæta icterohæmorrhagiæ* in the kidneys of field rats, and infected guinea pigs with the organisms (Stokes, A., Ryle, J. A., and Tytler, W. H., Weil's disease (spirochætosis icterohæmorrhagica) in the British Army in Flanders, *Lancet*, 1917, i, 142). In the United States, Dr. Noguchi has confirmed the work by his finding of *Spirochæta icterohæmorrhagiæ* in domestic wild rats (Noguchi, H., *Spirochæta icterohæmorrhagiæ* in American wild rats, and its relation to the Japanese and European strains, *J. Exp. Med.*, 1917, xxv, 755).

<sup>3</sup> The determination of the animal species was made by Mr. Namiye, assistant in the Zoological Institute of the Imperial University at Tokyo, to whom we desire herewith to express our thanks.

specific differences in the immune serum proved that the spirochete found by us in the kidneys of house and wild rats is identical with *Spirocheta icterohæmorrhagiæ*.

We also examined six specimens of field mice.<sup>4</sup> In one instance we produced by intraperitoneal injection of mouse kidney emulsion, icterus and hemorrhages in the guinea pig, and identified numerous *Spirocheta icterohæmorrhagiæ* in the liver, though we found none by dark-field illumination in fresh kidney preparations of the field mouse. In five other cases, we could find no spirochetes, either by dark-field illumination or through intraperitoneal injection of kidney emulsion.

In our experiments with rats we employed the following method. A rat was permitted to bite a guinea pig in the hind leg. The rat was then killed, and a search was made by dark-field illumination for spirochetes in the blood, liver, and kidneys. When no organisms were found in this way, we injected intraperitoneally into the guinea pigs the blood, urine, liver, and kidney emulsion of the rat, awaiting the development of icterus and hemorrhages. As a rule, we examined three preparations by dark-field illumination, and if no organisms were found, we considered the result negative.

*Experiment 1. Kidneys.*—The number of rats employed was 92. In 26 of the animals, or 28.3 per cent, spirochetes could be demonstrated in the kidneys. We injected kidney emulsion from 8 of the 26 animals intraperitoneally into guinea pigs. 7 of these died on the 8th to the 13th day with marked icterus and hemorrhages; numerous organisms were found in their blood and liver. From 59 animals showing microscopically no organisms, we prepared kidney emulsion, which we injected intraperitoneally into guinea pigs; 5 (8.5 per cent) animals died with typical symptoms on the 8th to the 11th day. The number of spirochetes in the kidneys varied between one to a preparation and fifteen to sixteen in an optical field. Occasionally we found a tuft of spirochetes. All specimens showed brisk movements.

*Experiment 2. Urine.*—We examined the urine of 71 rats. In 22, or 31 per cent, spirochetes were present. In 19 rats, in which we found the organisms in the kidneys, they were contained also in the urine. In 3 out of 52 in which we were unable to demonstrate spirochetes in the kidneys, we discovered them in the urine. The urine sediment of 2 rats, in which no spirochetes had been found by either method, was injected intraperitoneally into guinea pigs. One

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<sup>4</sup> These mice were furnished us through the kindness of Dr. Miyakawas.

of the experimental animals died with typical symptoms on the 17th day. To test the virulence of the spirochetes, we then injected 0.1 to 0.2 cc. of urine containing spirochetes, from 5 rats, intraperitoneally into 5 guinea pigs. The animals died with marked icterus and hemorrhages 8 to 10 days later, thus proving a high degree of virulence for the organisms.

The spirochetes excreted in the urine appear for the most part in the nubecula, though some are found free. Their number fluctuates from fifteen to sixteen specimens in one nubecula to one or two in a preparation. The spirochetes in the nubecula are usually motionless, while the freely floating spirochetes make brisk movements. Occasionally degenerative forms are seen.

*Experiment 3. Blood and Liver.*—We examined the blood of 64 rats by dark-field illumination. In 20 of these we had found spirochetes in the kidneys and the urine, but we were unable to find them in the blood in a single instance. The intraperitoneal injections of guinea pigs with the blood of 6 rats, in which organisms were found in the kidneys, proved negative, as well as five blood injections from animals having no spirochetes in their kidneys. Similar experiments were conducted with liver emulsion and intestinal contents. We examined the livers of 62 rats, and the large intestines of 10. All these experiments were negative.

*Experiment 4. Rat Bite.*—As we had observed two cases of typical spirochætoxis icterohæmorrhagica following the bite of a rat, we proceeded to reproduce the conditions experimentally. Rats were permitted to bite guinea pigs in the leg. Of 50 experiments thus made, only one guinea pig died of icterus and hemorrhages on the 11th day following the bite. In this case, numerous *Spirochæta icterohæmorrhagiæ* were found in the blood and the liver. In two guinea pigs we observed in the blood another form of spirochete which Futaki and Ishiwara, with their associates, have assigned as the cause of rat-bite fever. It may be mentioned in passing that Drs. Kaneko and Okuda<sup>5</sup> of our clinic were able to confirm the view of the other Japanese investigators in this respect by finding the spirochetes in the kidneys of a patient dying from rat-bite fever. By our finding of spirochetolytic and spirocheticidal immune bodies in the serum of individuals who had recovered from rat-bite fever, we were able to affirm that the above mentioned spirochete is the causative agent of rat-bite fever.

Mizukuchi reported before the Pathological Anatomical Congress held in Tokyo in April, 1916, an interesting fact which grew out of an experimental investigation of rat-bite fever. He permitted guinea

<sup>5</sup> Kaneko, R., and Okuda, K., The distribution in the human body of *Spirochæta icterohæmorrhagiæ*, *J. Exp. Med.* 1917, xxvi, 325.

pigs to bite rats, and certain of the animals died later, as the result of a peculiar icteric and hemorrhagic condition. In the liver of these animals he found a spirochete which, according to our view, is identical with *Spirochæta icterohæmorrhagiæ*. Mizukuchi believed the spirochete to be the cause of rat-bite fever, a conclusion with which we cannot agree. It is highly probable that the microorganism was *Spirochæta icterohæmorrhagiæ*, and that it was communicated to the guinea pigs in the same manner as in our experiments.

The manner in which *Spirochæta icterohæmorrhagiæ* is conveyed by the bite of a rat to man or the guinea pig is not yet clear. As no spirochetes were found either in the mouth or the blood of the rat, it is not likely that they are carried directly through biting. It may be assumed, however, that they are conveyed indirectly from rat urine, with which the mouth of the rat may be contaminated, into the wound created by the bite of the rat.

As stated above, the spirochetes found in the kidneys and the urine of rats resemble in form and movement *Spirochæta icterohæmorrhagiæ*. When injected intraperitoneally into the guinea pig, the animal succumbs after a time, with icterus and hemorrhages, symptoms which are identical with those of spirochætosus icterohæmorrhagica. These facts seem to prove without a doubt that the spirochete in question is identical with *Spirochæta icterohæmorrhagiæ*, the causative agent of Weil's disease, but further experiments to confirm this point are cited below.

#### *Action of Spirochæta icterohæmorrhagiæ Immune Serum on Spirochetes Found in Rats.*

For Pfeiffer's tests we used guinea pig liver emulsion rich in spirochetes, and immune horse serum of *Spirochæta icterohæmorrhagiæ*. Control experiments were also carried out. These were made (1) with rat spirochetes, *Spirochæta icterohæmorrhagiæ*, and isotonic salt solution; and (2) with *Spirochæta icterohæmorrhagiæ* and its immune horse serum. The results are shown in Table I.

As shown in the table, no spirochetes were contained in the peritoneal fluid in from 30 minutes to 2 hours after injection in the main experiments conducted with rat spirochetes and immune horse serum,

TABLE I.  
Results by Pfeiffer's Method with Rat Spirochetes and Immune Horse Serum of *Spirochæta icterohæmorrhagiæ*.

Animal No.	Case No.	Intraperitoneal injections into guinea pigs.						No. of spirochetes in the peritoneal fluid.		Course of experimental animal.	Autopsy.	Spirochetes in liver.	
		Spirochetes.			Serum.								
		Generation.	No.	Amount injected.	Type.	Amount injected.	cc.	After 30 min.	After 2 hrs.	0 in one preparation. " " "	Well for 1 mo. " " "		
1	219	R 16/1 III Rat spirochetes.	10 in one field.	1	Horse serum.	1	2 in one preparation.	0 in one preparation.	Well for 1 mo.			+	
2	220	" " "	10 " "	1	" "	1	0 " "	" " "	" " "			+	
Control experiments.													
3	217	R 16/1 III Rat spirochetes.	10 in one field.	1	Sodium chloride.	1	3-6 in one field.	1-2 in field, lively.	Died 5th day, icterus and hemorrhage.	+	Numerous.	+	
4	218	Pure culture.	10 " "	1	" "	1	3-6 " "	1-2 " "	" 5th " "	+	"	+	
5	221	" "	10 " "	1	Horse serum.	1	1 in preparation, lively.	None in preparation.	Well for over 1 mo.			+	



and in an experiment made with *Spirochæta icterohæmorrhagiæ* and immune horse serum, while numerous spirochetes were found in the control experiments conducted with isotonic salt solution. These tests show that the serum of horses immunized with *Spirochæta icterohæmorrhagiæ* has a spirocheticidal and spirochetolytic action upon the spirochetes of the rat.

*Experiments to Determine Whether Goat Serum Obtained by Immunizing with Rat Spirochetes Will Destroy Spirochæta icterohæmorrhagiæ.*

We immunized a number of goats with a pure culture of the rat spirochete or with liver emulsion containing the organism, and were able to obtain an effective serum. We then made Pfeiffer's tests with the goat serum and *Spirochæta icterohæmorrhagiæ* and the rat spirochete in the same order as the previous experiments. Table II shows the results.

The result of these experiments shows that the goat serum obtained by immunizing with rat spirochetes is capable of destroying *Spirochæta icterohæmorrhagiæ*.

On the basis of these experiments, we may conclude that the spirochetes found in the kidneys of house and wild rats are identical with Inada and Ido's *Spirochæta icterohæmorrhagiæ*.

We examined the kidneys of 6 field mice and injected their kidney emulsion into the peritoneal cavity of guinea pigs. One of the animals died on the 25th day of icterus and hemorrhages, but here the period of incubation was too long, and the hemorrhages were insignificant. As up to the present we have not observed stall infection among the guinea pigs, we must conclude that though the field mouse harbors our spirochetes in the kidneys, they are not of great virulence in that animal. Miyajima's findings also are in accord with our observations, for he observed in the kidneys of field mice spirochetes which appeared to be far less virulent than those observed by us in the kidneys of house and wild rats.

TABLE II.  
*Pfeiffer's Tests Made with Spirochæta icterohæmorrhagicæ and Immune Goat Serum from Rat Spirochetes.*

Animal No.	Case No.	Intraperitoneal injections into guinea pigs.				No. of spirochetes in the peritoneal fluid.				Course of experimental animal.	Autopsy.	Spirochetes in liver	Results.
		Spirochetes.		Serum.		After 30 min.	After 2 hrs.						
		Generation.	No.	Amount infected.	Type.			Amount infected.					
									cc.				
1	223	R 30/1 IX Rat spirochetes.	10 in one field.	1	Goat.	1	0 in two preparations.	0 in two preparations.	Well for over 1 mo.			+	
2	224	B.-C. IX 682 S. <i>icterohæmorrhagicæ</i> .	10 " "	1	"	1	0 " "	0 " "	" " 1 "			+	
3	225	" " "	10 " "	1	"	1	1-2 in specimen,* moving slowly.	0 " "	" " 1 "			+	
Control experiments.													
4	226	B.-C. IX 682 S. <i>icterohæmorrhagicæ</i> .	10 in one field.	1	Sodium chloride.	1	2-3 in one field, lively.	1 in one field, lively.	Died 5th day, icterus and hemorrhages.	+		Numerous.	
5	229	R 30/1 IX Rat spirochetes.	10 " "	1	" "	1	2-3 " "	1 " "	" " 5th " "	+		"	

\* Specimen covered about 70 optical fields, Leitz oc. 3,  $\frac{1}{2}$  oil immersion.

## DISCUSSION.

1. In 34 out of 92 cases, or 37 per cent, spirochetes were present in the kidneys or in the urine, as demonstrated directly by dark-field illumination and indirectly by inoculation.

2. The form, movement, virulence, and immune serum prove that our spirochete is identical with *Spirochæta icterohæmorrhagiæ*. The organisms cannot be demonstrated in the blood and the liver, but in the urine of rats harboring *Spirochæta icterohæmorrhagiæ* in the kidneys, they are present without exception.

3. Urine containing spirochetes, even in small amounts (0.1 to 0.2 cc.) infects guinea pigs when injected intraperitoneally.

4. *Spirochæta icterohæmorrhagiæ* are rarely conveyed directly to the guinea pig by the bite of the rat.

5. In Japan, the rat is undoubtedly a carrier of the causative agent of spirochætosis icterohæmorrhagica. *Mus decumanus* was found to be a carrier in 40.2 per cent of 149 cases, *Mus alexandrinus* in 0.8 per cent of 24 cases.

Whether in addition to *Epymis norvegicus* and *alexandrinus* and *Microtus montebelloi* there are still other carriers of Weil's disease is not known.

*The Transmission of Spirochæta icterohæmorrhagiæ Infection by the Rat and the Soil.*

It is noteworthy that our spirochete appeared only in the kidneys of rats—not in their blood and liver. This peculiarity of distribution is observed also in man during the convalescent stage of Weil's disease and in the guinea pigs treated with immune serum. As already stated, when the antibodies have been fully developed, the spirochete remains only in the kidneys. As, however, the rats examined by us were apparently in a good state of health, notwithstanding the numerous spirochetes found in their kidneys, we may assume that the organisms do not cause any, or only a slight degree of illness. The probability is that, entering the animal by the mouth or skin, after a time they find their way into the kidneys.

We conducted a number of experiments on mice and white rats, injecting 14 mice with 0.5 cc. of blood or liver emulsion from an

infected guinea pig. Four of the animals died with icterus, the others remaining well. Dr. Kaneko found spirochetes only in the kidneys. Of four white rats, one died of icterus; here also spirochetes were found only in the kidneys. It is possible that ordinary rats possess a greater degree of resistance to the infection than mice and white rats.

The behavior of the spirochete within the rat is open for further study, but we know that the rats harboring spirochetes always excrete them in the urine. The organisms thus find their way to the ground, where they may infect other rats as opportunity offers. In all probability they are disseminated by means of rats, the soil and the animals forming a circle of habitation for the spirochetes. It happens rarely that human beings are infected directly through the bite of rats, the infection being usually transmitted from the soil, where evidently the excreted spirochetes lodge and thrive. On these grounds we can explain the epidemics of spirochætosis icterohæmorrhagica which occur in coal mines and among the farmers in the vicinity. Rats are constant tenants of the mines, and it is known that the miners go barefoot. A similar statement may be made concerning the transmission of Weil's disease on the battle-fields of Europe. There the rats living in the trenches infect the soldiers.

It has been stated that the spirochetes of the field mice are less virulent than those of house and wild rats. This fact does not seem to harmonize with the infection of farmers in the fields; but on the other hand, it must be mentioned that wild rats are also found in the fields to some extent. It was certainly very striking that cooks and maids working in kitchens showed so high a percentage of spirochætosis icterohæmorrhagica lesions. Of 84 patients admitted to our clinic, 23 were in occupations which subjected them to contact with rats; *i.e.*, 8 were cooks, 6 maids, 3 pastry cooks, 3 "kamaboko" (bone meal) manufacturers, 2 vegetable dealers, and 1 fish dealer. The clinical observations alone made it probable that there was some connection between the rat and spirochætosis icterohæmorrhagica, though cases in which the infection was traced directly to the bite of a rat are rare. Brief protocols of the histories of two such patients are given below.

As shown in the table, the percentage of rats harboring spirochetes is high in those regions where Weil's disease is prevalent, while it is low in regions free from that disease. But it must be remembered that the number of rats examined from the latter regions was small, and that the difference may be attributable to this cause. Miyajima

reported that he found *Spirochæta icterohæmorrhagiæ* in the field mice of a region free from Weil's disease, and it is possible that the rats of a healthy region may also harbor the spirochetes in their kidneys and excrete them with the urine.

The life conditions of the spirochetes seem to be manifold. It was brought out by epidemiological studies that a certain degree of moisture in the soil and a certain temperature are necessary for proliferation. Damp coal mines particularly are favorable places of infection, while the disease is relatively rare in coal mines that are dry. As far as temperature is concerned the optimum in the cultivation of the spirochetes is 22–25°C. Weil's disease in Japan occurs but rarely in the height of summer and the coldest part of winter, but mostly at the end of spring, in early summer, and particularly in the autumn. In coal mines, which have an even temperature, the disease is equally prevalent at all seasons of the year.

That the soil plays an important part in the life of cholera bacilli was emphasized by Emmerlich; and it is known that not only cholera bacilli, but other bacteria and protozoa as well fail to thrive in an acid soil. Our spirochetes also die in a weakly acid medium. This fact led us to trace a connection between the endemic appearance of spirochætosis icterohæmorrhagica and the composition of the soil. Through the kindness of Dr. Takaishi, a member of the Agricultural Institute, we were able to obtain information concerning the distribution of acid, alkali, and neutral soils in the Province of Fukuoka. Comparing the spread of spirochætosis icterohæmorrhagica with the chemical condition of the soil, we discovered the interesting fact that on the whole, the disease occurs rarely in regions having an acid soil, while it is endemic in alkali and neutral soils. The composition of the water and soil of coal mines was also found to vary, an alkali reaction being obtained from the soil of the Ita, Hondo, and Nishizinmachi coal mines, while the earth of the Miike, Yamano, and Akaike mines showed an acid reaction. In Miike no cases of spirochætosis icterohæmorrhagica are known to occur, while in the course of 18 months over 300 cases occurred in Ita, and in Hondo annually from 80 to 100 are recorded. Although there was a high percentage of rats harboring spirochetes in Akaike (80 per cent), only 7 or 8 cases of the disease occur there per year. In the Yamano mine the yearly number is from 5 to 20 cases.

## CONCLUSIONS.

1. On the basis of these findings, we conclude that the extermination of rats and field mice is a highly important prophylactic measure against Weil's disease.

2. The chemical composition of soil and water plays an important part in the development of *Spirocheta icterohæmorrhagicæ*, and consequently in the spread of the disease of which it is the causative agent.

We desire to express to Professor Ryokichi Inada our appreciation of the guidance which he has given to our work.





## THE CLINICAL ASPECTS OF SPIROCHÆTOSIS ICTERO-HÆMORRHAGICA OR WEIL'S DISEASE.

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### *Stages of the Disease.*

Fiedler<sup>1</sup> has divided the progress of Weil's disease into three stages. According to him, the first 2 or 3 days constitute the initial period. The second stage commences variously from the 3rd to the 6th day following the onset of the disease, and is characterized by icterus, edema of the liver, tumor of the spleen, albuminuria, hemorrhagic diathesis, etc. He classes a defervescent period from the 7th to the 8th day as the third stage, and adds also a stage of convalescence. We believe, however, that a different division of the disease into three periods, *i.e.*, first or febrile stage, second or icteric stage, and a third or convalescent stage, has better justification. As will be explained in detail, each stage has its characteristic features with respect to the behavior of the spirochetes in the blood, the antibodies, the excretion of the organisms with the urine, and their distribution in the organs. According to our view, the first and second stages continue each for about a week; and the convalescent stage begins with the 3rd week of illness, although the boundaries of the different stages are not sharply demarcated. Icterus, the main symptom of the second stage, has its beginning in the middle of the first and reaches its climax in the second period.

*Febrile Stage.*—This stage continues from the onset of the disease to the 6th or 7th day. The main symptoms, which are initiated with chills or high fever, are intestinal disturbances, headache, cramp-

<sup>1</sup> Fiedler, A., Weitere Mittheilungen über die Weil'sche Krankheit, *Deutsch. Arch. klin. Med.*, 1892, 1, 232.

ing muscular pains, marked hyperemia of the conjunctiva bulbi, albuminuria, etc. Death occurs rarely in this stage. The period is characterized by free circulation of the spirochetes in the peripheral blood, although their number may not be great. Blood taken during this period and injected intraperitoneally into guinea pigs produces a typical reaction. The infectivity of the blood decreases gradually, as shown by the 69 infection experiments cited in Table I.

TABLE I.

*Infection Experiments with Blood from Weil's Disease.  
April, 1912, to December, 1915.*

Day of illness.	No. of animals injected.	Positive.	Per cent positive.
2	4	4	100.0
3	10	10	100.0
4	13	13	100.0
5	12	11	91.6
6	14	12	85.7
7	8	4	50.0
8	4	0	0
9	1	1	100.0
12	1	0	0
18	1	0	0
19	1	0	0
Total..... 69			

As shown in the table, guinea pigs were in all cases infected in a typical manner when they received intraperitoneally blood drawn from patients during the first 4 days of illness. By the 5th day, the infectivity of the blood is already diminished, one case only out of the 12 proving negative (positive 91.6 per cent). With blood taken on the 6th day the results showed 85.7 per cent positive, and on the 7th day 50 per cent. These findings seem to indicate that with the progress of the disease, the spirochetes disappear gradually from the blood stream, owing to the spirochetolytic and spirocheticidal action upon them of antibodies developed in the blood. We are led to believe that antibodies, though few in number, are present as early as the 5th day, but their number is not sufficient for demonstration by Pfeiffer's method.

No difference is observed in this stage of Weil's disease in the peritoneal fluid obtained after the use of serum of patients and that of healthy persons. The guinea pigs under experimentation die of typical symptoms on the same day as the control animals, or a day later or earlier. Hence the inference of a slight development of antibodies can be made only indirectly from results achieved with the infection experiments.

Spirochetes are excreted with the urine during this stage, and the injection of the urinary sediment produces in guinea pigs a typical infection. It is not as a rule possible, however, to demonstrate the organisms in the urine by dark-field illumination. The distribution of spirochetes in the organs resembles that found in the experimental animals. Numerous spirochetes are seen in the liver.<sup>2</sup>

*Second or Icteric Stage.*—This stage continues from the 7th or 8th to the 12th or 13th day of illness. Generally it covers a little less than a week. As a rule, the symptoms of the first stage decrease in intensity, and in their place appear icterus, hemorrhagic diathesis, marked general weakness, nervous symptoms, and cardiac weakness. But all these symptoms have their onset during the middle or toward the end of the febrile period, and reach their greatest intensity during the second stage. Death is most prevalent during this period. Of eighteen fatal cases, in sixteen death occurred between the 8th and the 16th day from the onset of the disease.

This stage is characterized by the fact that it is rarely possible to infect guinea pigs by the intraperitoneal injection of patients' blood, only one out of six experiments proving positive. It is evident that the spirochetes have already disappeared from the peripheral blood. Moreover, it is possible to demonstrate antibodies in the blood by Pfeiffer's method. The finding of spirochetes in the peritoneal fluid of the experimental animals by Pfeiffer's method shows that a different condition exists in them from that observed in the control animals. Spirochetolysis is present, and the experimental animals die in a typical manner 4 or 5 days later than the control animals. When on the other hand, the injected human serum contains

<sup>2</sup> Kaneko, R., and Okuda, K., The distribution in the human body of *Spirochæta icterohæmorrhagiæ*, *J. Exp. Med.*, 1917, xxvi, 325.

no antibodies, the experimental animals die on the same day as the control animals, or at the utmost 1 or 2 days earlier or later. A difference of 3 days is rare. Hence we conclude that when the experimental animals die 4 or more days later than the control animals, the serum must contain a certain number of antibodies. Their degree of development at this stage is incomplete, for with complete development of antibodies, the experimental animals would recover from the infection.

In our experiments we employed 1 cc. of serum and 1 cc. of pure culture of the spirochetes, or a liver emulsion containing the organisms; ten spirochetes to an optical field by dark-field illumination (Leitz oc. 3, obj.  $1\frac{1}{2}$  oil immersion). Injections were made intraperitoneally. The gradual increase in the number of antibodies can be observed by testing, according to Pfeiffer's method, serum obtained during various stages of the disease. The duration of life of the experimental animals lengthens in the course of the disease, and finally with complete development of antibodies, the animals do not become ill at all. Occasionally the antibodies are fully developed as early as the 8th day. We observed two cases of this kind.

In the second stage, the spirochetes are easily demonstrated in the urine by dark-field illumination. On the 10th day of illness it was possible to show them in 17.4 per cent of the cases, with a gradually growing percentage up to 52.2.

Corresponding to the development of the antibodies, the spirochetes disappear first from the blood, and then from the liver. Hence their distribution in the second stage differs from that of the febrile stage.

*Third or Convalescent Stage.*—This period begins on the 13th or 14th day. The intensity of the icterus characteristic of the second stage then subsides gradually, and anemia and marked emaciation become apparent. This period is characterized by complete development of the antibodies in the blood, the disappearance of spirochetes from the blood, their abundant excretion with the urine, frequent high fever (called relapsing fever by Weil, and after fever by us), and later on, by the excretion of antibodies with the urine. The distribution of the spirochetes in the organs is noteworthy. No organisms are found in the liver and other organs except the kidneys,

where they are always present. They are occasionally found in cardiac muscle.

The percentages of spirochetes excreted with the urine gradually increase and reach their maximum on the 15th or 16th, up to the 23rd or 24th day of illness. By the 19th or 20th day practically all cases show spirochetes in the urine. After the 25th day; the percentages decrease. It was found that twenty-two out of twenty-four patients had ceased to excrete spirochetes after 40 days. One patient excreted them on the 42nd day, and another on the 63rd. The duration of most abundant excretion covers from 3 to 6 days. Comparing the onset of the period of abundant excretion of the spirochetes in the urine with the appearance of the complete antibodies in the blood, we find that the first phenomenon precedes the latter by from 2 to 5 days, though occasionally the conditions may be reversed.

Histologically, the spirochetes are found on the 17th or 18th day only in the kidneys, having disappeared from the other organs with complete development of antibodies in the blood.

#### *Incubation Period.*

The period of incubation, according to our computations, varies from 5 to 7 days with skin infection; it is seldom as long as 13 days. In the epidemic of Weil's disease which occurred in Hildesheim, Germany, Hecker and Otto estimated the time of incubation as covering at least a week. Our observations in Japan entirely coincide with this finding, and our conclusions concerning the incubation period are based on the histories of patients following our study of the portal of entry of the *Spirochæta icterohæmorrhagiæ* in animals.

#### *Pathology of the After Fever.*

The behavior of the fever during the first stage coincides with that observed in cases of Weil's disease in Europe. During the period of convalescence, frequently there is a recurrence of rather high, remittent fever, which Weil and Fiedler have termed relapsing fever. According to Fiedler, in Europe it occurs in 40 per cent of the cases. In Japan we have observed it in 28.2 per cent of our

patients. 'So far as the character of this fever is concerned, our interpretation differs from that of Weil and Fiedler, and we have employed the term after fever for the following reasons.

The fever has its onset on the 14th or 15th day, sometimes on the 13th, rarely on the 12th or 16th day. There may be an afebrile interval of from 2 to 10 days following the fever of the first stage. The fever covers a period of from 4 to 20 days. It usually reaches a height of 38-40°C., and the temperature is frequently above that of the first stage. The fever is markedly remittent in character, particularly at its maximum. The temperature rises gradually, remains from 3 to 4 days at its greatest height, and then begins gradually to decline. In fatal cases, there is no after fever.

As indicated above, we cannot agree with the view of Weil and Fiedler that this fever is to be regarded as relapsing in character, on the following grounds.

(a) We have never observed a recurrence of the main symptoms; *i.e.*, hyperemia of the conjunctiva bulbi, exacerbation of the icterus, hemorrhagic diathesis, edema of the lymph glands, etc. Notwithstanding the presence of the fever at this time, only the symptoms which usually accompany a rise in temperature are found, such as headache, general weakness, etc.; and although the temperature is high, life is not endangered. The fever of the first stage is accompanied by marked leukocytosis, while this condition is not constant in the later fever.

(b) Secondly, all the infection experiments conducted on guinea pigs during this period were negative. With the exception of the kidneys, no spirochetes, or very few, are found in the organs.

(c) In the third place, the spirochetolytic and spirocheticidal antibodies are fully developed in the blood at this period. If, on the other hand, spirochetes were to reappear in the blood, we should be justified in regarding this fever as relapsing.

We believe, therefore, that the fever of the third stage is different in character from that of the first.

The pathogenesis of the after fever is not clear. Clinically, no particular changes are observed in the organs, and there are no complications or suppurations. The blood of eight patients which was subjected to careful bacteriological examination was found to be

sterile. Hence we are unable to trace the origin of the fever to a secondary infection. It appears that the after fever coincides with the presence of the antibodies in the blood and the abundant excretion of spirochetes in the urine. Furthermore, the curve of the serum-treated guinea pigs which survived resembled closely the fever curve of patients in the third stage of illness.

It is our belief that this fever is to be regarded as a reaction on the part of the immune organism to the subsequent resorption of spirochetic toxins. Spirochetolysis continues for the whole period of the disease, but only with the appearance of complete immunity does the organism react in the form of fever. The toxins arise from the disintegration of the spirochetes within the organs, above all, the kidneys, where probably a proliferation of spirochetes takes place during the convalescent stage, when numerous specimens can be observed. On the other hand, it must be remembered that the after fever occurs in only 28.2 per cent of the cases, while the phenomena of antibody formation and excretion of spirochetes occur with constancy. It appears that the pathogenesis of the after fever requires still further investigation.





# A CONTRIBUTION TO THE ETIOLOGY AND PATHOLOGY OF RAT-BITE FEVER.\*

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PLATES 32 TO 34.

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Although rat-bite fever in Japan was recognized a long time ago by Miyake and others as a definite entity, and its symptomatology has been described in detail, we know little of its pathological anatomy. According to Ishiwara only two postmortem examinations have been reported, one by Blake, and another by Miura and Toriyama.

Our knowledge of the causative agent of rat-bite fever is of recent date. Futaki and his associates reported in their first communication the finding of a long spirochete, measuring 9 to 10  $\mu$  in the lymph glands on the 10th or 13th day of illness, and on the 33rd day in tissue fluid obtained from the bitten area. Those found in the lymph exudate had small waves, those from sections of lymph glands, numerous steep waves. Later on they discovered in man, in the wound of the rat bite, and in the blood of mice which had received injections of patient's blood, shorter, thick spirochetes, 2 to 6  $\mu$  long. These had regular, close, and steep waves, and a filament at each end. As these long and short spirochetes are morphologically distinct, and both have been demonstrated in rat-bite fever, the question arose whether they are of the same species, or whether rat-bite fever may be caused by two different spirochetes.

The problem could not be solved at the time by Futaki and his coworkers. In the meantime, Ishiwara and his associates found in their investigations of experimental rat-bite fever spirochetes of the shorter variety, which were morphologically identical with the short variety of Futaki. Kitagawa and Mukoyama, as well as Midzukuchi, reported finding these spirochetes in experimental rat-bite fever.

We performed recently a postmortem examination on an individual who had rat-bite fever in typical form, and were in a position to make

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definite anatomical studies. We found in the tissues certain spirochetes which will be described, and were able to confirm the work of Futaki and his associates, who were the first to designate these spirochetes as the cause of rat-bite fever. We have furthermore come to the conclusion that the long type of spirochetes found by them previously and the short variety discovered later in experimentally infected animals are not diverse in type, but belong to the same species of spirochete.

### *Spirochetes in the Tissues.*

We examined the blood of a patient having rat-bite fever during the afebrile interval, guinea pigs inoculated with the patient's blood, as well as the swollen lymph glands, but found no spirochetes. Later Ido and other authors found in blood films taken at the height of the disease, a number of the short type of spirochetes.

*Distribution in the Organs.*—We focused our attention particularly upon the suprarenals and the kidneys, in view of the fact that Ishiwara and his associates in their experiments on guinea pigs found the organisms mainly in the suprarenals, and also because *Spirochæta icterohæmorrhagiæ* is present in these organs in the convalescent stage of Weil's disease. After repeated careful examinations, we discovered numerous organisms, particularly in the kidneys.

The spirochetes in the kidneys are located almost exclusively in the casts and cylindroids of the straight tubules, in the canals of Henle, and the intercalary portion of the boundary layer. They were never found free in the lumen and the interstitial spaces. In the suprarenals, we observed the organisms in the cortical cells. In addition, we found in the interstitial space of the testicle a specimen of the short spirochete. Other organs, *i.e.*, the liver, lymph glands, spleen, lungs, intestines, stomach, bladder, prostate, heart, and brain, showed no spirochetes; nor could any be found in the skin of the bitten area, treated with Orth's fluid, and in the swollen lymph glands.

The total number of spirochetes found in the organs was somewhat over 100 in 30 preparations; most of these were contained in renal casts and cylindroids. In the suprarenals, we observed seven specimens in ten preparations, and in the testicle one in several preparations. Their distribution is very irregular; in the densest regions five to six specimens were found in one cast, and twenty-eight in a preparation.

*Forms of the Spirochete.*

As shown in Fig. 1, the forms of the organism are various. The spirochete may be long or short, thick or thin, rigid or pliable, smooth or ragged. The waves may be regular or irregular, gradual or abrupt, many or few. The diversity of characteristics is so great as to lead to the belief that many kinds of spirochetes are present. They can, however, be classified according to size into long and short, with intermediary forms between the two groups.

The short spirochetes found by us are from 1.7 to 5.0  $\mu$  in length. As a rule, they have close and regular waves, from two to six steep waves to a specimen, are relatively thick, with smooth edges, and stain deeply when impregnated with silver. Occasionally one finds at one or both ends, fine thread-like extensions which stain somewhat less deeply. These regularly waved spirochetes are identical with the short spirochete of Ishiware. But a large number of the short spirochetes are more rigid in form, have irregular waves, and are not smooth at the edges. These are atrophic abortive forms. The spirochetes found in blood films have regular waves.

The long type spirochete measures 6.0 to 10.0  $\mu$ , and has numerous, small, steep, irregular waves. These specimens are not smooth and regularly formed, and appear rigid.

We examined the mouse preparations (from the injection of patient's blood) sent us by Futaki, and are convinced that his short type of spirochete is identical with our own, with the exception that our specimens were somewhat thinner and more rigid, which may be due to the fact that our strain was taken from man. Futaki's long type, which we observed in great numbers in his preparations, is morphologically identical with ours.

In addition we made careful histological examinations of guinea pigs infected by Ido and his collaborators through rat bites. The spirochetes found in these animals are morphologically identical with our short spirochete.

## DISCUSSION.

In Weil's disease, the distribution of *Spirocheta icterohæmorrhagiae* varies with the different stages of illness. At the beginning the organ-

isms circulate in the blood, while in the convalescent stage, following the complete development of immune bodies, they can be demonstrated only in the kidneys. A similar distribution seems to prevail in rat-bite fever. In the experimental animals, the spirochetes circulate in the peripheral blood. That identical conditions exist in the human body is proved by the fact that mice and guinea pigs can be infected by the inoculation of patient's blood. At the height of the disease, the short spirochete, though not numerous, has been observed microscopically in the blood. In our case we found the spirochetes located mainly in the kidneys, although at the height of illness they could be demonstrated also in the peripheral blood. The localization within the kidneys is undoubtedly the usual order in later stages of the disease.<sup>1</sup>

Inada was the first to suggest the formation of antibodies in rat-bite fever. He was followed by Ido and his associates of our clinic, who proved the truth of the assumption. Judging by analogy with Weil's disease, we suggest that the irregular distribution of spirochetes in the kidneys may be attributable to the partial destruction that has taken place owing to the action of the antibodies.

The characteristics of our spirochetes—their irregularity and rigidity—may also be considered as the result of the action upon them of the immune bodies. We know that in *spirochætosis ictero-hæmorrhagica* the organisms disappear in the course of the illness through the action of the spirochetolytic and spirocheticidal antibodies, leaving various irregular so called degenerated forms of spirochete. It is highly probable that this is also the case in rat-bite fever in man. On the other hand, in the animal body, where no specific immune bodies are developed, the spirochetes exist in their typical forms as found by Futaki (short spirochete) and also by Ishiwaru. In conformity with this, we have our finding of short spirochetes in the blood of patients at the height of the disease.

Finally we are confronted with the important problem as to whether the short and long spirochetes constitute two different species or one species of spirochete. Futaki seems to regard them as organisms different in type, and for that reason was not clear

<sup>1</sup> At the time of death the patient had passed the height of the disease.

concerning the etiology of rat-bite fever. On the basis of histological investigations, we conclude that these two forms of spirochete belong to the same species, as one type grades into the other, morphologically speaking. Moreover, it does not seem probable in our case of rat-bite fever that spirochetes of different types simultaneously infected the same individual and localized in the renal casts.

The long spirochetes occur almost exclusively in human tissues and we may regard them as old or degenerated forms which have become irregular and stiffly bent. The short spirochetes, on the other hand, which have regular waves are the typical, young specimens. They can be found in the blood of patients having rat-bite fever, as well as in the experimental animals.

Futaki and his collaborators, 2 weeks prior to the appearance of our publication, on the basis of experiments made with pure cultures, arrived at a conclusion identical with our own.

### *Pathological Anatomy.*

*Case 1.*—Z. I., male, age 70, locomotive engineer. Clinical diagnosis: rat-bite fever.

*History.*—A month previously the patient while asleep was bitten by a rat, in the forefinger of the left hand. The wound bled. After 2 weeks the area became swollen and red; very sensitive. Incision was made. The following day the patient observed blotches, the size of a finger, in rows on the left forearm. These disappeared gradually, only four remaining. Similar spots appeared in the left cubital region and upper arm, and along the tendon of the forefinger. 20 days after the bite, the patient observed swelling of left axillary lymph glands, which became as large as a pigeon's egg, and were very painful. Temperature was not taken, but after the incision there was fever. A few days ago, slight chills, fever, headache, and loss of appetite, which have continued.

Admitted to clinic, Oct. 18, 1915.

*Present Illness.*—Male, medium height. Nutrition fair. Conjunctivæ, mucosa of palate, and pharynx markedly hyperemic. Tongue swollen and coated. Lymph glands in left axilla, size of bean to pigeon's egg; very sensitive. Both cubital glands swollen to size of lentil; elastic, soft, not sensitive. Inguinal glands not swollen. Thickened arterial walls. Liver palpable two fingers below costal arch, not sensitive. Spleen not palpable. No ascites. Urine negative.

Left forefinger shows scar 1 cm. at point of incision. Joint somewhat affected. Skin on dorsal side of left second metacarpophalangeal articulation somewhat eroded and scarred. Surrounding area red and hot. On dorsal sur-

face of left hand, a number of reddened areas, size of quarter, somewhat raised, also along radial side of fore and upper arm. The spots are united by fine red stripes.

*Blood*.—Oct. 20, with ascending fever, hemoglobin (Sahli), 60 per cent, erythrocytes, 4,012,000, leukocytes 5,600. Oct. 29, interval; hemoglobin 64 per cent, erythrocytes 4,290,000, leukocytes 4,200. Nov. 2, rising fever, erythrocytes 3,090,000, leukocytes, 12,100.

*Course*.—Death from senile anorexia, 7 weeks after admission. Altogether eight fever attacks, each lasting 3 to 4 days, with afebrile intervals of 2 to 3 days. At onset of illness, typical fever; later, atypical, often remittent. Height of fever in an attack 38.5–39.5°C., once reaching 40.1°C. (Text-fig. 1).

Left axillary glands at first swollen to size of pigeon's egg, diminished gradually. Oct. 30, when excised, they were the size of a bean. Gland was flat and elastic, partly hyperemic, grayish white. Spots on skin became gradually fainter; none found on Oct. 26. Gradual loss of weight. Died of inanition and marasmus, Dec. 6.

*Autopsy*.—9 hours later. Body poorly nourished. Rigor mortis only in knee joints. Skin dry, dark brown. Scar on left forefinger. No edema or exanthemata. Peritoneum contained no abnormal fluid. Mesenteric lymph glands swollen, on section somewhat injected. Pericardium contained a table-spoonful of serous fluid, with fibrin fragments; inner surface of pericardium opaque. Heart in diastole, size of patient's fist, weight 265 gm. In left ventricle, no fluid or coagulated blood; in right ventricle, small mass of fatty coagulum. Pericardium somewhat opaque, endocardium without luster. Intima of root of aorta, marked arteriosclerotic spots; valves negative. Myocardium soft, yellowish brown. Spleen somewhat soft, small, 8.0 by 7.0 by 1.5 cm., 64 gm., capsule corrugated, trabecular. Lungs, marked anthracosis; hyperemic; scattered atelectatic foci. Glands of hilum somewhat swollen. Kidney, left, 1.2 by 5.0 by 3.0 cm., 116 gm., capsule adherent, surface not smooth, partly nodular and granulated, congested. Large cysts scattered on surface. On section hyperemic, cortex thin; columns of Bertin yellowish, granulated; mucous membrane of pelvis slightly hyperemic, somewhat enlarged. Parenchyma friable and cloudy. Right kidney, 11.0 by 4.5 by 2.5 cm., 98 gm., similar to left. Suprarenals hyperemic. Liver, 30.0 by 13.5 by 5.5 cm., 1,062 gm. Surface smooth, rib furrow on right lobe. Parenchyma on section yellowish, dull, but acini recognizable; congested, consistency tough. Gall bladder contained greenish mucous gall; mucosa negative. Stomach contained small quantity of mucus; mucosa hyperemic and opaque. The same may be said of mucosa of duodenum; papilla duodeni permeable. Pancreas, hyperemic. Intestines, mucosa opaque and hyperemic; follicles not swollen. Bladder contained small amount of turbid urine; marked hyperemia of mucosa. Prostate slate-colored, not hypertrophied. Cranium, subdurally, large mass of serous fluid; meninges soft, somewhat opaque and hyperemic; brain slightly hyperemic. Bone marrow of tibia negative.

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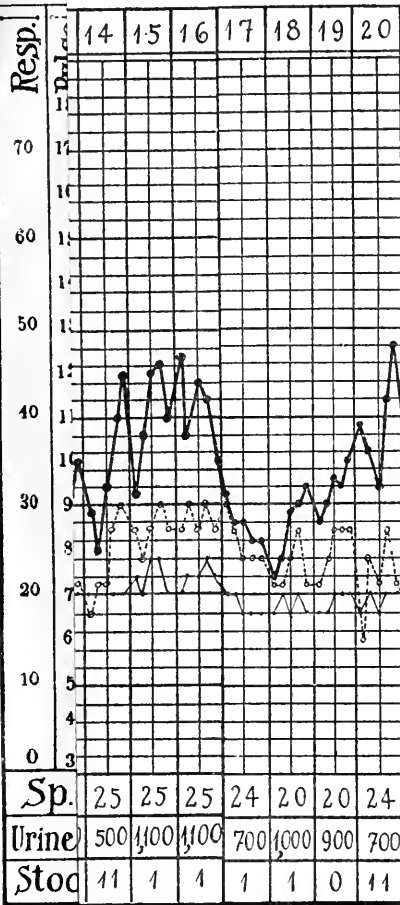
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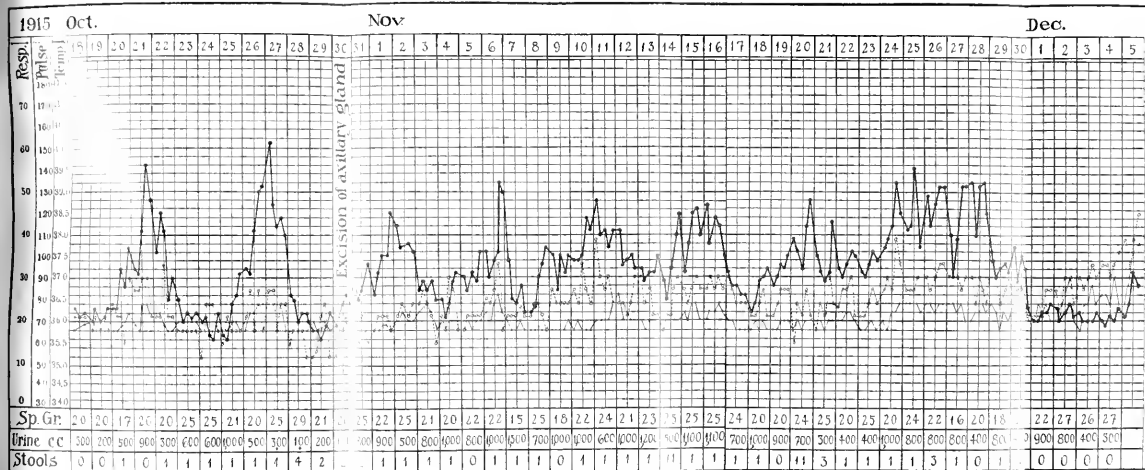
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TEXT FIG. 1. Case 1, male



*Diagnosis.*—Rat-bite fever. (1) Marasmus universalis. (2) Degeneratio parenchymatosa cordis, hepatis, renum. (3) Gastroenteritis catarrhalis. (4) Cystitis catarrhalis. (5) Edema and hyperemia meningeae. (6) Arteriosclerosis gradus gravioris. (7) Nephritis interstitialis chronica.

*Histological Examination. Lymph Glands.*—From left axilla, removed Oct. 30, negative. Stained with hematoxylin and eosin. Showed simple hyperplasia of adenoid tissue. In capsule and its surrounding area, infiltration with plasma cells and leukocytes. Parenchyma markedly hyperemic, but showed no regressive or marked inflammatory changes.

The other organs were placed in fixing solution, and stained. The following is a report of the hematoxylin and eosin preparations.

*Kidney.*—By low power, hyaline degeneration of glomeruli, interstitial proliferation, and casts, as well as cylindroid formation in the tubules. Cortex and medulla hyperemic here and there. Subcapsular lobulation of cortex, consisting of scar tissue, containing degenerated hyaline glomeruli and the remains of kidney tubules; marked cellular infiltration. By high power, marked changes in kidney tubules, particularly of the convoluted tubules. Cells markedly enlarged, partly degenerated, nuclei remaining unstained and cells necrotic, staining faintly reddish violet. Some cells when stained with Sudan show small fat globules. In other places, marked desquamation and destruction of cells, with stained nuclei. Slighter changes in wall epithelium of lower sections of convoluted tubules, Henle's loops, and the intercalary portion; nuclei stain well; no necrosis, though sometimes marked destruction and desquamation of epithelium. Infiltration of fat globules more marked in lower sections of the tubules. The epithelium of convoluted tubules contains very small fat globules resembling lipoid substance, while in the epithelium, of straight tubules, infiltration of larger fat globules is common.<sup>2</sup> The renal casts and cylindroids are always hyaline, and rarely contain blood cells and desquamated epithelium. Numerous urinary casts particularly in the lower sections of the tubules, and in convoluted tubules, Henle's loops, intercalary portion, etc. Now and then in the enlarged tubules one finds a round, hyaline body composed of layers. The medullary canals also contain numerous casts. The glomeruli show total or partial hyaline degeneration, some being hyperemic. No abnormal fluid in Bowman's capsules. The interstitial spaces are markedly dilated, but without new connective tissue or cellular infiltration, generally edematous, and in part transformed into hyaline. The blood vessels show no marked changes.

*Liver.*—Cells stain faint reddish violet with hematoxylin and eosin, often contain fat globules, fine vacuoles, and yellowish brown pigment bodies in the neighborhood of the nuclei. No marked swelling or opacity of liver cells. With Sudan III, the cells in acini centers show infiltration with small fat globules, while

<sup>2</sup> This phenomenon was also observed by us in spirochaetosis icterohæmorrhagica.

in the cells of the acini periphery there is an accumulation of large drops of fat. The central parts of the acini are broken up and markedly degenerated. The cells are atrophic, disintegrated, or necrotic; many have disappeared. The gaps are filled with blood cells; various grades of necrobiosis in cells. Nuclei are unstained or disintegrated; some have disappeared. Marked fatty degeneration. Liver cells of acini periphery are relatively compact. Stellate cells of Kupffer slightly enlarged; in part fatty degeneration has taken place. Some contain blood cells and pigment granules. The interstices show no marked changes. Slight infiltration of round cells here and there. In the bile duct nothing noteworthy. Wall epithelium rarely shows infiltration of fat globules.

*Spleen*.—Negative. Follicles not enlarged, trabeculae well developed. Scattered hyperemia. Central arteries thickened, intima shows hyaline degeneration. The nuclei of scattered pulp cells do not stain well; destroyed. No large necrotic foci, cellular infiltration, pigment deposits, erythrophagocytosis, or microorganisms.

*Suprarenals*.—Show in deep layer of cortex circumscribed hyperemia, causing slight scattering of cells.

*Cardiac Muscles*.—Somewhat pale and indistinct; no fatty or other degenerations present. In the fibers near the two ends of the nuclei, numerous pigment bodies, which stain yellowish with Sudan III (brown atrophy).

*Lungs*.—Catarrhal changes in consolidated parts.

*Mesenteric Glands*.—Blood vessels in the medulla are markedly dilated and filled; lymph sinuses dilated. Endothelium, desquamated and degenerated, often taking up erythrocytes. Some scattered degenerated cells in sinus and follicles. Mucosa of gastrointestinal tract covered with mucus; epithelium desquamated, and cellular infiltration in propria.

Muscle fibers of gastrocnemius here and there slightly swollen; partly changed into hyaline. No marked destruction of fibers or fatty degeneration.

*Nerve Cells*.—Cells of brain cortex and spinal cord here and there are slightly enlarged or destroyed; some are pyknotic, nuclei not stained. Nerve fibers show, though rarely, slight swelling and disintegration.

*Skin*.—Shows scar at site of bite.

*Case 2*.—R. H., female, age 40, peasant woman. Admitted to Second Medical Clinic, July 28, 1908.

*History*.—70 days ago, while asleep, the patient was bitten in the upper lip by a rat. The wound bled, but healed in a few days. After a month, the skin of the bitten area became red, swollen, and very sensitive. Swelling and redness extended gradually to the angle of the mouth on the right side, and then to the submaxillary region. Since that time, patient had attacks of chills and fever, with headache and malaise. First attack lasted for a week, followed after an interval of 1 week by a second. Patient observed also about ten scattered spots, varying from the size of a finger point to a walnut, on the extremities and the chest. High fever with nausea, vomiting, loss of appetite, headache, etc.

Swelling of lip and redness subsided gradually. Fever came in attacks, often accompanied by vomiting.

*Present Illness.*—Medium height and nutrition. At left angle of mouth, a reddish brown spot, 2 cm. in width. Neck in front shows numerous spots, some connected. Extremities also show spots, 1 to 4 cm. in width. Over the entire body, scattered exanthematous areas, which disappear on pressure. Tongue coated. Pulse 120, regular, tense. Breasts and abdominal organs negative. Biceps and brachialis anticus somewhat sensitive. No albumin in urine. No edema. Temperature 38.8°C.

*Course.*—July 30. Blood: leukocytes 14,333, erythrocytes 3,495,000. Aug. 8. Rising fever, up to 38.7°C. Vomiting. Aug. 10. Muscular pains in lower extremities. Aug. 12. Disappearance of spots and redness of mouth region. Aug. 15. Vomiting. Aug. 18. Rapid pulse. Aug. 20. Fever, 39°C., increased pulse (150 per minute), regular, small. No urination, marked meteorism. Aug. 22, unconscious. Death by cardiac insufficiency.

*Autopsy.*—12 hours after death. Height 150 cm., weight 36.4 kg. Nutrition poor. Rigor mortis only in knee joints. Diffuse postmortem discoloration on back and neck. Abdomen somewhat distended. Skin pale and dry, no exanthemata, no edema. In abdominal cavity about 100 cc. of clear yellowish fluid. Intestines moderately distended with gas. Serous membranes of intestines pale, slate-colored. Diaphragm, height of fourth intercostal space. No abnormal fluid in thorax. Lungs, no adhesions. Pericardium contained about a tablespoonful of clear yellow fluid; inner surface of pericardium somewhat injected. Heart, size of patient's fist. In right ventricle a large amount and in left ventricle moderate amount of dark red fluid blood and fatty coagulum. Epicardial fat tissue moderate, somewhat edematous. Endocardium smooth, myocardium somewhat opaque. Consistency of heart soft. Intima of root of aorta shows in the region of the aortic valves a number of yellow plaques. Left lung voluminous. Lower edge of lower lobe shows hypostatic induration. On section, congested. Bronchial mucosa markedly hyperemic. Peribronchial glands partly swollen and markedly anthracotic. Right lung moist and congested, resembling left. Spleen, 12.0 by 5.0 by 2.0 cm., weight 58 gm. Capsule wrinkled. On section congested, follicles and trabeculae visible, consistency soft. Left kidney, 11.5 by 6.0 by 2.5 cm., 117 gm. Capsule easily removed. On outer surface, urinary cyst the size of a pea. Parenchyma shows slight opacity. Columns of Bertin somewhat swollen, parenchyma yellowish, boundary of both substances partly indistinct. Right kidney, 11.3 by 6.0 by 2.8 cm., 121 gm., resembling the left. Liver, 24.0 by 16.0 by 6.0 cm., 1,340 gm. Surface smooth. On section somewhat yellowish and opaque. Gall bladder contained dark green gall. Mucosa negative. Stomach contained a large mass of sour smelling grayish green food. Mucosa in spots hyperemic. Intestines, small, held a large mass of dark yellowish green contents. Mucosa slate-colored or greenish yellow, somewhat edematous; scattered small nodules the size of pin-head.

These nodules are dark brown, somewhat coarse, and yellowish in the center. Lower section of small intestine contained twenty-four specimens of *Ascaris lumbricoides*; mucosa markedly injected. Large intestine contained small fecal (fluid) mass; mucosa somewhat injected. Retroperitoneal and mesenteric lymph glands not markedly swollen. Urinary bladder contained small amount of turbid urine; mucosa opaque and injected. Mucosa of vagina also somewhat injected. Portio uteri red. Mucosa of uterus slightly hyperemic.

*Diagnosis.*—Rat-bite fever. (1) Marasmus universalis. (2) Degeneratio parenchymatosa cordis, hepatis, renum. (3) Gastroenteritis catarrhalis. (4) Cystitis catarrhalis. (5) Hydrocele tubæ dextræ. (6) Ascaridosis.

No microscopic examination.

*Case 3.*—N. H., female. Examination of swollen axillary gland excised at beginning of illness showed marked hyperplasia of parenchyma cells. The follicular substance is particularly hyperplastic. In places, the hemorrhages in the follicles are marked, leaving small islands of cells between. No necrosis of the parenchyma. In the peripheral zone of the gland there is quite a dense accumulation of polynuclear leukocytes. Corresponding with the site of hemorrhage, the epithelium shows here and there desquamation and degeneration. Erythrophagocytosis is present, but not as plainly as in spirochaetosis ictero-hæmorrhagica. In the medullary substance, scattered leukocytes are found.

*Case 4.*—Skin preparation (old) from area bitten by rat. Corium and subcutaneous tissue markedly hyperemic, edematous, and infiltrated with polynuclear leukocytes. No abscess formation. Corium tissue shows hemorrhages and fibrin deposits. No changes in epidermis. Scattered necrotic cells in infiltration zone, but no extensive necrotic foci.

#### SUMMARY AND DISCUSSION.

Of the two postmortem examinations on rat-bite fever previously reported, the patient of Miura and Toriyama, a woman, aged 32 years, died on the 70th day after the onset of the disease. Anatomically there were no marked changes. Histological examinations were not made. Macroscopically, the following findings are reported: increase of cerebrospinal fluid, hyperemia of the meninges, inflammatory edema of the lungs, cloudy swelling of the liver, etc.

The other, Blake's case, was a woman of 67, who died 1 month after the bite of a rat. The anatomical findings were acute ulcerative endocarditis, subacute myocarditis, interstitial hepatitis, glomerular and interstitial nephritis. The suprarenals contained a large amount of perivascular fluid. There were infarcts in the spleen and kidney. In general, the organs were hyperemic; the lung showed hemorrhages and edema. Histologically, Blake found marked degeneration, necrosis, and infiltration in the cardiac muscles, infiltration of polynuclear cells, and increase in the interstices of the liver, also marked polynuclear infiltration of the shrunken kidney.

Blake was able to demonstrate *Streptothrix muris rattii* in the tissues, which he assigned as the cause of rat-bite fever. He viewed the degeneration and infiltration in the organs, together with the ulcerative endocarditis, as the result of the streptothrix infection.

### *Pathological Anatomy.*

Our Case 1 was that of a man of 70, who died 80 days after the bite of a rat, and 2 months from the onset of illness. In contrast to Blake's case, there were no marked pathological changes, with the exception of the parenchymatous changes in the organs. The kidney changes are attributable in part to the condition of shrunken kidney, which had existed for some years previous to the infection. Other changes, such as the marked hyperemia, swelling, and degeneration of the tubular epithelium, must be regarded as caused by the rat-bite fever. In part, the formation of casts can likewise be so interpreted. The liver shows marked changes, degeneration, necrosis, and destruction of liver cells, particularly in the acinus centers, with hyperemia and hemorrhages, the fatty degeneration of the liver cells being especially marked in the acinus centers. Parenchymatous changes of the liver and kidney were observed macroscopically in Case 2.

The hematopoietic organs, *i.e.*, the spleen, lymph glands, and the bone marrow, show in general no abnormalities. The local lymph glands at the beginning of illness show marked hyperplasia of the parenchyma cells. In this, the part played by the follicular substance must be taken into consideration. The cellular hyperplasia is simpler and of an inflammatory character. Although the hyperemia, hemorrhages, and the loosening of the follicles are distinct, there are no noteworthy regressive changes. There is a slight catarrhal condition of the sinus and erythrophagocytosis, while in spirochaetosis icterohæmorrhagica these manifestations are marked. In the course of the disease, the swelling of the local lymph gland subsides gradually, and the corresponding changes become less. Other lymph glands, such as the mesenteric, also show slight swelling, with hyperemia and catarrhal changes.

In addition we noted in our case catarrhal changes in the mucous membrane of the stomach, cystitis catarrhalis, congestion of the

lungs, etc. The degeneration of the muscle and nerve cells, though slight, must be regarded as a phenomenon of this disease. The meninges were also hyperemic and edematous.

We have few comments to make on the exanthemata, as we lacked material for anatomical investigation. The site of the bite showed, as in the fourth case, cellular infiltration, edema, and degeneration (acute exudative inflammation). In the lungs, as already mentioned, we found signs of congestion, but the accompanying marked inflammatory changes must be regarded as the result of a mixed infection, because we found an accumulation of diplococci.

The changes observed by Miura and Toriyama agree with ours, but a great difference exists between Blake's case and ours. In his case there were marked infiltration of pus cells and regressive changes in the organs—even an ulcerative endocarditis. These differences cannot be explained on the basis of the duration of illness. Though the time of illness in our case was very long; it was marked up to the time of death by fever. In Blake's patient, we must attribute the marked changes, as emphasized also by the author, to streptothrix infection. If this streptothrix is the cause of rat-bite fever, the changes which have been described would be significant, but in view of our findings in Japan, and on the basis of the histological studies made by Ido, Ito, Wani, and Okuda,<sup>3</sup> there is doubt in our minds whether the streptothrix should be so regarded. It is possible that Blake's case was one of mixed infection.

In their experiments with rat-bite fever, Ishiware and his associates found in addition to swelling of the bitten area and lymph glands, acute inflammatory changes in the kidney, and hyperemia and hemorrhages in the suprarenals. We examined the guinea pigs of Ido and his associates, which were infected by the bite of rats; and also observed swelling of the bitten area and of the regional lymph glands, with slight nephritis, hyperemia of the lungs, catarrh of the mucosa of the stomach, and slight parenchymatous degeneration in the organs. These changes on the whole resemble those of human cases.

<sup>3</sup> Ido, Y., Ito, H., Wani, H., and Okuda, K., Circulating immunity principles in rat-bite fever, *J. Exp. Med.*, 1917, xxvi, 377.

In conclusion we desire to express to Dr. Ryokichi Inada our thanks for his assistance in this work, to Dr. H. Nakayama of the Pathological Institute of the Imperial University in Kyushu for permitting us to use the protocols, and to Dr. Takeya of the Second Medical Clinic of the University for the use of patients' histories.

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#### EXPLANATION OF PLATES.

##### PLATE 32.

FIG. 1. Various forms of spirochetes found in the tissues. From silver preparations.

##### PLATE 33.

FIG. 2. Spirochetes found in renal casts. From silver preparations.

##### PLATE 34.

Spirochetes in the tissues.

FIG. 3. Spirochete in cortex of suprarenal.  $\times 1,130$ .

FIGS. 4 to 6. Spirochetes in urinary casts. Fig. 4,  $\times 630$ . Fig. 5,  $\times 380$ . Fig. 6,  $\times 460$ .





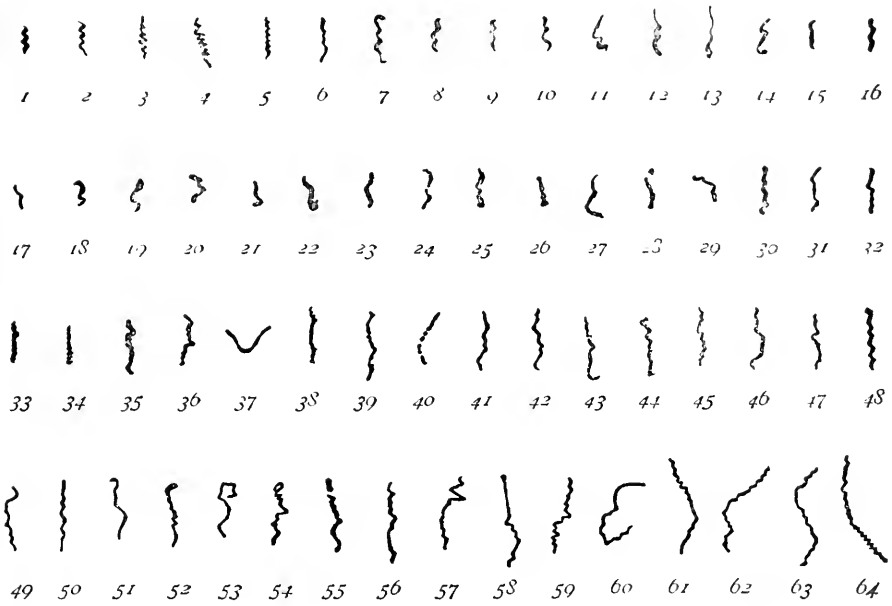


FIG. 1.

(Kaneko and Okuda: Rat-bite fever.)





FIG. 2. (Kaneko and Okuda: Rat-bite fever.)



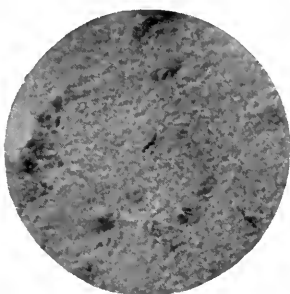


FIG. 3.

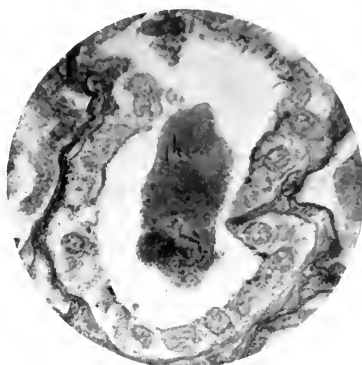


FIG. 4.



FIG. 5.

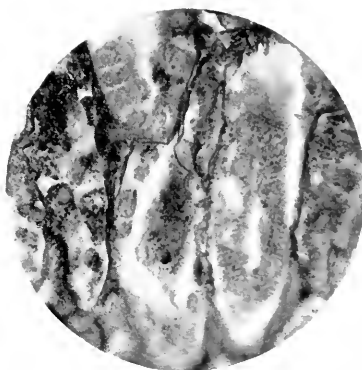


FIG. 6.

(Kaneko and Okuda: Rat-bite fever.)



## CIRCULATING IMMUNITY PRINCIPLES IN RAT-BITE FEVER.\*

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(Received for publication, February 20, 1917.)

We owe our knowledge of the causative agent of rat-bite fever largely to the researches of the Japanese workers, Futaki and Ishiwara, and their associates. Futaki in 1915, reported finding a spirochete in preparations from the bitten area and the lymph glands of persons having rat-bite fever. Ishiwara and Ohtawara, during the same year, conducted histological studies on guinea pigs which had been experimentally infected with rat-bite fever, and found a similar spirochete. In 1916, numerous other authors in Japan took up the problem which centered particularly in the variety of types of spirochetes found in the disease in man and the experimental animals. Two main groups—long and short spirochetes—were distinguished. In May, 1916, Futaki announced on the strength of cultural studies made by him and his associates, that the two types are in all probability identical, and a little later he definitely advanced the belief that the organism is the causative agent of rat-bite fever. His work was confirmed by Kaneko and Okuda,<sup>1</sup> who reported on the pathological anatomy of the disease.

The short spirochete found by Futaki, and Kaneko and Okuda, coincides with respect to form and movement with that observed by Ishiwara and his associates in experimentally infected guinea pigs. These spirochetes have been identified in guinea pigs, white rats, and mice, following the intraperitoneal injection of patients' blood and emulsion of lymph gland. In the guinea pig, fever resulted. In no instance were these spirochetes found in healthy guinea pigs, white rats, or mice. They were also absent from the blood and tissues of infected animals receiving salvarsan treatment.

On the basis of the facts above cited we may say that in all probability the spirochete which has been described is the causative agent of rat-bite fever. The premises are, however, not complete, for we

\* Published in *Tokyo Iji-Shuho*, September, 1916, No. 1990.

<sup>1</sup> Kaneko, R., and Okuda, K., A contribution to the etiology and pathology of rat-bite fever, *J. Exp. Med.*, 1917, xxvi, 363.

lack one significant proof in order to make the assertion conclusive. It is necessary to demonstrate in the serum of patients having rat-bite fever an immunity principle which is specific for the spirochetes of that disease. Up to the present time such confirmation has been lacking.

In the postmortem examination made in our clinic of a patient having rat-bite fever, the spirochetes were found located mainly in the kidneys, and not in other tissues. A similar localization of spirochetes in the kidneys is true in the convalescent stage of spirochæ-tosis icterohæmorrhagica, and as that phenomenon, together with the disappearance of symptoms in Weil's disease, has been explained on the ground of the formation of antibodies in the blood, we may assume that a similar condition exists in rat-bite fever; *i.e.*, antibodies are present in the blood of those who recover from the disease. In order to prove this point, we obtained blood serum from three patients recovering from rat-bite fever, and conducted the experiments cited below.

The spirochetes employed by us came from guinea pigs, the animals having been infected through the bite of the rat, *Mus decumanus*. Ishiwara has affirmed that these organisms are morphologically identical with those studied by him and obtained from experimentally infected animals. We used no spirochetes taken directly from human beings.

#### *Histories of Patients from Whom Blood Was Obtained for Animal Experiments.*

*Case 1.*—Male, age 17, farmer. In Aug., 1915, the patient was bitten in the tip of the right little finger by a house rat. After 2 weeks, the bitten area became swollen and red, followed by swelling of right neck and axillary glands, and typical fever. Admitted to First Surgical Clinic of the University. Diagnosis, rat-bite fever. Discharged, cured, after 59 days. Blood taken on July 27, 1916.

*Case 2.*—Male, age 40, farmer. The patient was bitten in the upper arm by a rat, in Feb., 1916. After 20 days, the patient developed high fever, chills, swelling and sensitiveness of right axillary glands; typical exanthematous spots. Admitted to First Surgical Clinic. Treated with salvarsan. Blood taken on Aug. 4, 1916.



*Case 3.*—Female, age 35, wife of merchant. In May, 1916, the patient was bitten on the right upper eyelid, by a house rat. After 3 weeks, swelling and redness of bitten area; eye closed. High fever, swelling of neck glands, and exanthematous spots. Admitted to the Dermatological Clinic. Diagnosis, rat-bite fever. Blood taken on Aug. 7, 1916.

### *Dark-Field Preparations.*

We examined by dark-field illumination a mixture of one loopful of guinea pig blood containing one to two spirochetes to an optical field and one loopful of serum from recovered cases of rat-bite fever. Control experiments were made with serum obtained from recovered cases of Weil's disease or beri-beri, and isotonic salt solution.

In the control experiments we found relatively numerous spirochetes, but we were unable to identify any actively moving organisms in the experiments made with the spirochetes and serum of rat-bite fever, although motionless or very inactive specimens were present. Here the spirochetes had been almost totally destroyed. We found only one or two specimens in a preparation.

Experiments were then undertaken to ascertain the efficacy of diluted serum. For this purpose we employed serum in isotonic salt solution, diluted two-, four-, and eightfold, and made tests similar to those which have been described. Twice diluted serum still showed the spirocheticidal and spirochetolytic properties, but with serum in a dilution of four, no such effects were observed.

It is evident from these experiments that the serum of patients convalescing from rat-bite fever contains a specific immunity principle against the spirochetes of that disease, although the immune body is relatively weak.

### *Pfeiffer's Phenomenon.*

Pfeiffer's tests were made with the rat-bite fever serum and the blood of guinea pigs containing the spirochetes. In view of the fact that the immune bodies which develop in the blood in rat-bite fever are relatively weak, as demonstrated by the experiments described above, we employed in these tests two parts of serum to one part of

blood, in order to obtain conclusive results. The quantities used were 2 cc. of serum and 1 cc. of heart blood taken from an experimentally infected guinea pig. This mixture was injected into the peritoneal cavity of another guinea pig. After 30 minutes and again after 2 hours peritoneal fluid was drawn by puncture, and a search for spirochetes was made by dark-field illumination.

The experimental animals were also kept under observation, in order to throw further light on our results, and two series of control experiments were carried out. In the one case, we made Pfeiffer's tests with the serum of beri-beri or spirochaetosis icterohæmorrhagica; in the other, we injected 1 cc. of the infected guinea pig blood but no serum. The results are shown in Tables I, II, and III.

It will be seen that no spirochetes were found in the peritoneal fluid taken 30 minutes or 2 hours after injection in Pfeiffer's tests made with the immune serum of rat-bite fever and the blood of guinea pigs containing the rat-bite fever spirochetes. On the other hand, the peritoneal fluid contained numerous, briskly moving spirochetes in the control experiments made with beri-beri or spirochaetosis icterohæmorrhagica serum, as well as in the experiments where only 1 cc. of guinea pig blood, and no serum, was injected. It is clear that the serum of persons recovering from rat-bite fever has a specific spirochetolytic and spirocheticidal effect upon the organisms in question.

The blood of the experimental animals kept under observation was examined by dark-field illumination; in the case of the animals injected from Case 1 on the 12th, 20th, and 29th day after injection; of animals receiving blood from Case 2, on the 7th, 15th, and 24th day; and animals infected from Case 3, on the 5th, 13th, and 22nd day. The results in these experiments were similar to those of Pfeiffer's phenomenon tests. In the case of the guinea pig showing positive spirochetolysis, no spirochetes could be detected in the peripheral blood, while in the control animals numerous organisms were found 5 days after injection, thus proving the efficacy of the serum in protecting the guinea pig against rat-bite fever infection.

TABLE I.  
Case I.

Guinea pig No.	Injections into peritoneal cavity.		Spirochetes in peritoneal fluid.		Further course of guinea pigs found in blood.		
	Patient furnishing serum.	No. of guinea pig furnishing blood. Quantity.	After 30 min.	After 2 hrs.	12th day.	20th day.	29th day.
1	H. 2 cc. rat-bite serum, filtered.	1 cc. blood, R 27/16 V (1 spirochete in one field).	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.
2	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
3	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
4	" " "	1 cc. blood, R 30/16 V (1 spirochete in two fields).	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
5	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
Control experiments.							
6	R. 2 cc. beri-beri serum.	1 cc. blood, R 32/16 V (1 spirochete in two fields).	1 in one specimen.*	1 in one specimen.	5-6 in one specimen.	2-3 in one specimen.	5-6 in one field.†
7	" " "	" " "	2-3 " " "	1-2 " " "	2-3 " " "	1-2 " " "	3-4 " " "
8	" " "	" " "	2-3 " " "	1-2 " " "	Died on 4th day, diarrhea.		
9	" " "	" " "	1 " " "	1 " " "	Killed on 4th day, diarrhea.		

\* Cover-glass preparation, 70 optical fields, Leitz oc. 3, obj.  $\frac{1}{2}$  oil immersion.† Optical field, Leitz oc. 3, obj.  $\frac{1}{2}$  oil immersion.

TABLE II.  
Case 2.

Guinea pig No.	Injections into peritoneal cavity.		Spirochetes in peritoneal fluid.		Further course of guinea pigs found in blood.		
	Patient furnishing serum. Quantity.	No. of guinea pig furnishing blood. Quantity.	After 30 min.	After 2 hrs.	7th day.	15th day.	24th day.
10	E. 2 cc. rat-bite serum, filtered.	1 cc. blood, R 31/16 V (more than 1 spirochete in field).	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.
11	"	"	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
12	"	"	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
Control experiments.							
13	K. 2 cc. beri-beri serum.	1 cc. blood, R 34/16 V (more than 1 spirochete in field).	4 in one specimen.*	3-4 in one specimen.	1-2 in one specimen.	1 in three to four fields,†	2 in one field.
14	"	"	1-2 " " "	2-4 " " "	1 in two to three specimens.	1 in one field.	4-5 " " "
15	"	"	4 " " "	4-5 " " "	1-2 in one specimen.	1 in one to two fields.	1 in one to two fields.
16	"	"	5-6 " " "	4 " " "	1-2 " " "	1-2 in one field.	3-4 in one field.

\* Cover-glass preparation, 70 optical fields, Leitz oc. 3, obj.  $\frac{1}{2}$  oil immersion.† Optical field, Leitz oc. 3, obj.  $\frac{1}{2}$  oil immersion.

TABLE III.

Case 3.

Guinea pig No.	Injections into peritoneal cavity.		Spirochetes in peritoneal fluid.			Further course of guinea pigs found in blood.			Spirochetes
	Patients furnishing serum. Quantity.	No. of guinea pig furnishing blood. Quantity.	After 30 min.	After 2 hrs.	5th day.	13th day.	22nd day.		
17	G. 2 cc. rat-bite serum, filtered.	1 cc. blood, R 29/16 V (1-2 in one field).	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 " "
18	" "	" "	0 " "	0 " "	0 " "	0 " "	0 " "	0 " "	0 " "
19	" "	" "	0 " "	0 " "	0 " "	0 " "	0 " "	0 " "	0 " "
Control experiments.									
20	H. 2 cc. spirochaetosis icterohaemorrhagica serum, filtered.	1 cc. blood, R 30/16 V (2 in one field).	5-6 in one specimen.*	6 in one specimen.	1 in two to three specimens.	5-6 in one specimen.	3-4 in one field. †		
21	" "	" "	7 " "	5 " "	" "	2-3 in one field.	2-3 " "		
22	" "	" "	7-8 " "	7 " "	" "	Died.	1-2 in one field.		
23		1 cc. blood, R 29/16 V (1-2 in one field).	5 " "	6 " "	" "	7-8 in one specimen.			

\* Cover-glass preparation, 70 optical fields, Leitz oc. 3, obj.  $\frac{1}{2}$  oil immersion.† Optical field, Leitz oc. 3, obj.  $\frac{1}{2}$  oil immersion.

*The Action of Rat-Bite Fever Serum on the Spirochetes Circulating in the Blood.*

We injected 1 to 3 cc. of serum from the three cases of rat-bite fever intraperitoneally, intravenously, or subcutaneously into guinea pigs. The results were wholly negative, as the spirochetes in the circulating blood were not affected. We believe, however, that the negative character of the experiments is due to the small quantity of serum employed, and that in order to obtain a decisive result it is necessary to use for injection into the experimental animals serum in quantities corresponding to the total amount of blood employed.

As the guinea pig requires a large amount of serum on account of its size, we used mice in our further experiments. The maximum body weight of a mouse is 10 gm., and hence the amount of serum needed is not large; we found that 1 cc. of serum is effective in the mouse. This quantity of the immune serum of rat-bite fever was injected intraperitoneally into two mice, and intravenously into another. In the mouse receiving the intravenous injection, we were unable to find, 30 minutes after inoculation, any spirochetes in the blood by dark-field illumination, while numerous organisms had been detected prior to the injection. In the other mice, those which had received intraperitoneal injections, the number of spirochetes 30 minutes after inoculation was greatly decreased, only one or two specimens being found in a preparation after diligent search. It must be added, however, that in the latter case an increase in the number of spirochetes in the blood took place 1 or 2 days later. In the intravenously injected mouse, a small number of spirochetes could be detected in the blood on the 3rd day following the injection.

SUMMARY.

Summarizing the results that have been cited, we have proved that the blood serum of convalescents from rat-bite fever contains antibodies which are specific against the causative agent of that disease. The serum of rat-bite fever was capable of destroying the spirochetes not only in the hanging drop preparations, but also in the peritoneal cavity of guinea pigs. The guinea pigs employed for

Pfeiffer's test always remained well. In the experimentally infected mice receiving intravenously or intraperitoneally serum equalling in quantity the amount of infected blood, the numbers of spirochetes were greatly decreased or they disappeared for a definite period. It is not yet clear how long after recovery from rat-bite fever the antibodies are effective in the blood of human beings, and further investigations are needed to elucidate this point. In our experiments we found that serum taken from Case 1 showed definite spirochetolytic and spirocheticidal properties 11 months after the onset of the disease. In Case 2, the period was 6 months, and in Case 3, 3 months following the onset of rat-bite fever.

#### CONCLUSIONS.

1. The serum of persons who have recovered from rat-bite fever contains an immune body which destroys the spirochetes of that disease.

2. The experiments here cited confirm the findings of Futaki and his associates, with respect to the spirochete which is identical with the organism discovered by Ishiwara and Ohtawara and advanced as the causative agent of rat-bite fever. The etiology of rat-bite fever has, therefore, been definitely established.

3. The immune body present in blood serum during the convalescent stage of rat-bite fever is not equal in efficacy to that contained in the serum of spirochætoxis icterohæmorrhagica.

We take pleasure in expressing our appreciation of the direction and support which Dr. Ryokichi Inada has given to our work.





## PULMONARY EMBOLISM.

### AN EXPERIMENTAL STUDY.

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PLATES 35 AND 36.

(Received for publication, April 4, 1917.)

Virchow<sup>1</sup> was the first to recognize embolism and to study it experimentally. Cohnheim<sup>2</sup> was the next investigator to consider the subject. Cohn<sup>3</sup> produced a classical work on embolism in which he considered the subject in its broad aspects both clinically and experimentally. Welch<sup>4</sup> presented the more recent complete review of the subject, and the clinical and pathologic aspects of pulmonary embolism have been discussed by Wilson,<sup>5</sup> who suggested this investigation.

Many experiments by various investigators have been performed on embolism. However, the main purpose of most of the experimenters has been either to determine the location of the emboli or to study the method by which the lung becomes infarcted. Very few have aimed to determine the mechanism producing death.

Deaths due to pulmonary embolism may be divided into three groups:<sup>6</sup> (1) Immediate death occurring when only a small portion of the pulmonary circulation is obstructed. (2) Death caused within a few minutes and due to a complete or almost complete blocking of

<sup>1</sup> Virchow, R. L. K., Ueber die Verstopfung der Lungenarterie, *Notizen Gebiete Natur.-u. Heilk.*, 1846, xxxvii, 26-31.

<sup>2</sup> Cohnheim, J., Untersuchungen über die embolischen Processe, Berlin, 1872.

<sup>3</sup> Cohn, B., Klinik der embolischen Gefässkrankheiten, mit besonderer Rücksicht auf die ärztliche Praxis, Berlin, 1860.

<sup>4</sup> Welch, W. H., Thrombosis and embolism, in Allbutt's System of medicine, London, 1899, vi, 155-285.

<sup>5</sup> Wilson, L. B., Fatal post-operative embolism, *Ann. Surg.*, 1912, lvi, 809-817.

<sup>6</sup> Schumacher, Quoted by Meyer, W., The surgery of the pulmonary artery, *Tr. Am. Surg. Assn.*, 1913, xxxi, 223-248.

the pulmonary circulation. (3) Delayed death, the result of an increase by thrombosis of an initial blockage by an embolus of a portion of the pulmonary circulation.

The cause of death in either Group 2 or Group 3 is very evident. The mechanism by which death is produced by an embolus which blocks only a small part of the pulmonary circulation (Group 1) is unknown.

The present investigation was made for the purpose of determining this unknown factor, a purpose I have not been able to accomplish as it has been possible to produce death experimentally only by a more or less complete blocking of the pulmonary circulation. However, a brief report of the experiments may be of value.

All experiments, unless otherwise stated, were performed under ether anesthesia, and the carotid blood pressure and respiration were recorded. The emboli were sent into the venous circulation through the right femoral vein except in a few experiments in which the left external jugular was used.

The emboli employed were of two kinds. One kind was made of paraffin with a melting point of about 43°C. It was found that ordinary Christmas candles offered ideal material for these. By using the different colors it was possible to make each embolus distinctive and thus to tell definitely the relationship between the time the embolus was sent into the circulation and the position in which it was found at autopsy. Furthermore, the melting point of the candle was such that it became soft and would readily mold at body temperature but did not form droplets.

The other kind of embolus was made from the animal's own blood. The left external jugular vein and the right femoral vein were dissected free for a portion of their course. Blood vessel clamps were placed on them and the exposed portion of the veins was allowed to become distended with blood. It was then gently crushed with a hemostat, and after this a few cubic centimeters of tissue extract or blood serum from the same animal were injected into the damaged veins. Under these conditions large clots formed in the vessels very quickly. When the clamps were removed the clots were swept into the circulation, the process simulating the detachment of a thrombus in a patient.

The general results of all the experiments were the same. It was impossible to produce death or seriously imperil the life of the animal by emboli until the pulmonary circulation was greatly obstructed (Protocol 1). Some emboli passed from the femoral vein to a branch of the pulmonary artery without producing any effects on either blood pressure or heart beat. Usually, however, there was a slight drop in the blood pressure at the instant the embolus passed through the heart. This drop simulated that of a momentary inhibition of the heart. Section of the vagi, however, did not prevent it. In all probability it was due to a passage of the embolus through the pulmonary valves. This was quickly recovered from and the blood pressure usually maintained a practically uniform level until many emboli had been sent into the circulation. The first noticeable effect of the emboli was an increase in the venous pressure. The abdominal veins stood out prominently, and small veins severed in the operative procedure which did not bleed at the time of section began to bleed after the passing of a few emboli. Later, blood pressure decreased; in some experiments suddenly, in others it fell to zero slowly. The sudden drop was usually found to be due to a sudden blocking of the pulmonary artery, while in the gradual drop the emboli had blocked most of the pulmonary branches, and blood clots had formed around them. Respiration was unaffected until blood pressure began to decrease. Then it usually increased in both rate and amplitude. The blood pressure usually reached zero before respiration ceased.

At autopsy in every instance in which death had been produced by the emboli, the pulmonary circulation was found to be almost completely obstructed. Depending on the size of the emboli in some experiments, the pulmonary artery or the two branches were blocked; in others the occlusion occurred in the smaller branches. When the blood pressure had decreased slowly and venous pressure had increased considerably, many of the emboli sent into the circulation toward the end of the experiment were found in the right ventricle or vena cava.

The position assumed by the emboli in the pulmonary system in relation to the time of the injection was fairly uniform. As would be anticipated, the first emboli passed were usually found in the larger

branches of the pulmonary artery, and the first two or three emboli were found in the upper branch of the left branch of the pulmonary artery or in the pulmonary branch going to the largest lobe of the right lung. The positions of the rest of the emboli were never uniform.

As it was found impossible to produce sudden death by emboli in normal dogs without almost complete obstruction of the pulmonary circulation, the procedure was repeated using animals with a greatly depressed circulation. In a few experiments the animal was subjected to several hours' anesthesia before the emboli were used; other dogs were practically moribund with distemper. In all these animals the blood pressure was low (Protocol 2). The results in these experiments did not differ from those for which normal dogs were employed. Death was not produced until obstruction of the pulmonary circulation occurred.

It was deemed possible that general anesthesia was a factor. To obviate this, in a small series of animals the operative procedures were done under local anesthesia. The results were the same as when ether was employed (Protocol 3).

In a few experiments the emboli were sent in under sterile conditions. When very many emboli were employed the animal either died on the table or a short time afterward, or developed infarction of the lungs. When only a few emboli were employed the animal was not affected (Protocol 4).

Death from pulmonary embolism usually takes place in relatively strong patients at the time they attempt to leave the sickroom at the beginning of convalescence. They are usually active at the time of death. To simulate this condition a strong animal was fasted for several hours and the emboli passed into the circulation immediately after a period of intense exercise. The results of this experiment were also negative (Protocol 5).

## EXPERIMENTAL.

*Protocol 1.*—Mongrel, male; weight 7 kilos.

December 22, 1916. 8.45 a.m. Animal etherized. The apparatus was arranged to record carotid blood pressure and respiration. Right femoral vein exposed. Normal record taken, beginning at 9.10 a.m. Emboli of paraffin, 4 cm. in length and 0.5 cm. in diameter, were inserted into the right femoral vein as follows:

Time.	Embolus No.	Color of embolus.
<i>a.m.</i>		
9.21	1	Lavender.
9.22	2	Blue.
9.22½	3	Red.
9.23	4	Pink.
9.24	5	Yellow.
9.24½	6	Orange.
9.25	7	Green.
9.25½	8	Lavender and yellow.
9.26½	9	Green and yellow.
9.27	10	Red and yellow.
9.28	11	Mixed, several colors.

The blood pressure was affected as each embolus passed through the heart. Later, blood pressure began to decrease, the dog dying at 9.47 a.m. Fig. 1 gives the kymograph record of blood pressure and respiration, and Figs. 2 and 3 give the size and location of emboli.

*Protocol 2.*—Bulldog, female; weight 18.6 kilos.

December 27, 1916. The animal was in very poor condition. 9.40 a.m. Etherized. The apparatus was arranged to record blood pressure and respiration. A small amount of blood was withdrawn. The right common iliac vein was exposed, as was also the right femoral vein. A blood vessel clip was placed on the iliac vein, and after the contributory veins were gently crushed with a hemostat, blood serum taken from the blood which had been withdrawn was injected into the injured veins. The same process was repeated with the left external jugular vein. Clots soon formed in each vessel. 10.37 a.m. Blood pressure 60 mm. 10.44 a.m. The clamp was removed from the jugular vein and the clots were swept into the circulation. The blood pressure decreased 12 mm. but soon returned to normal. 10.48 a.m. The clamp was removed from the iliac vein. The blood pressure immediately fell and gradually decreased until it reached zero at 10.54 a.m. Fig. 4 gives the kymograph record of the blood pressure and respiration.

Autopsy was performed immediately. There was a large clot, 3.5 by 1 cm.,

in the pulmonary artery and extending into the right ventricle. This practically completely obstructed the artery. The left branch of the pulmonary artery was empty, but most of the terminal branches of the right branch of the artery were blocked with small blood clots.

*Protocol 3.*—Collie, female; weight 9.3 kilos.

May 24, 1916. The right femoral vein was exposed under local anesthesia with sterile technique. Ten emboli of paraffin, varying in size from 2.5 to 3.5 cm. long and 0.5 cm. in diameter, were inserted into the vein. The time used in putting the ten emboli into the circulation was 10 minutes. The animal showed no symptoms referable to the emboli. The pulse and respiration remained normal. The animal remained in good condition until May 27, when it developed a marked dyspnea and respiratory grunt. It was bled to death under ether.

Autopsy was performed immediately. The recent femoral wound contained a small hematoma but was not infected. The lungs contained many hemorrhagic areas of infarction measuring 0.5 to 2 cm. in diameter. On the upper anterior surface of the lower right lobe was a small area of marginal emphysema. It was possible to palpate the emboli in the pulmonary artery. There were no emboli in the heart. It was impossible to identify all the emboli as some were broken into two or more pieces. The right branch of the pulmonary artery was completely blocked with the exception of the very small branches. The main branch of the left branch of the artery was completely blocked. The center of each lobe of both lungs was necrotic.

*Protocol 4.*—Young mongrel, male; weight 9 kilos.

December 14, 1915. The animal was etherized and the right femoral vein exposed, with sterile technique. Four emboli, about 3 cm. long and 0.5 cm. in diameter, were inserted into the vein. The animal recovered quickly from the operation and has been in excellent health up to the present time, 15 months after operation.

*Protocol 5.*—Old bulldog, male; weight 8.15 kilos; very pugnacious.

March 27, 1917. The animal had been fasted for 80 hours previous to the beginning of the experiment. About 100 cc. of blood were removed and set aside to clot. Under local anesthesia the right femoral vein was exposed and clamped with a blood vessel clamp. After the vein had been traumatized with a hemostat some of the animal's own blood serum containing small clots was injected. In a short time the vessel was filled with clots. The animal was then exercised for 5 minutes, after which the clamp was removed from the vessel and the clots were swept into the circulation. The respiration and pulse did not change. The process was repeated with the left external jugular vein. These results were also negative. 1 hour later the animal was etherized and bled to death.

Autopsy was performed immediately. Many of the terminal arteries in every lobe of the lungs were filled with clots. It was estimated that about half of the pulmonary circulation had been occluded.

Figs. 5 and 6 are kymograph records obtained in two similar experiments.

## SUMMARY.

Emboli made of paraffin and the animal's own blood were sent into the venous circulation of dogs. Death did not occur until the pulmonary circulation was practically occluded. The results were the same whether the blood pressure of the animal was normal or depressed by ether or disease and whether the procedure was carried out under ether or local anesthesia.

## EXPLANATION OF PLATES.

## PLATE 35.

FIG. 1. Kymograph record of blood pressure and respiration in Protocol 1. Time in minutes and seconds. Normal blood pressure 90 mm. of mercury. Note the drops in the blood pressure as the emboli pass through the heart. The break in the record covers a space of 3 minutes. Note the increase in respiratory movements as the blood pressure falls.

FIG. 2. Photograph of emboli used in Protocol 1, which were recovered at autopsy. Actual size.

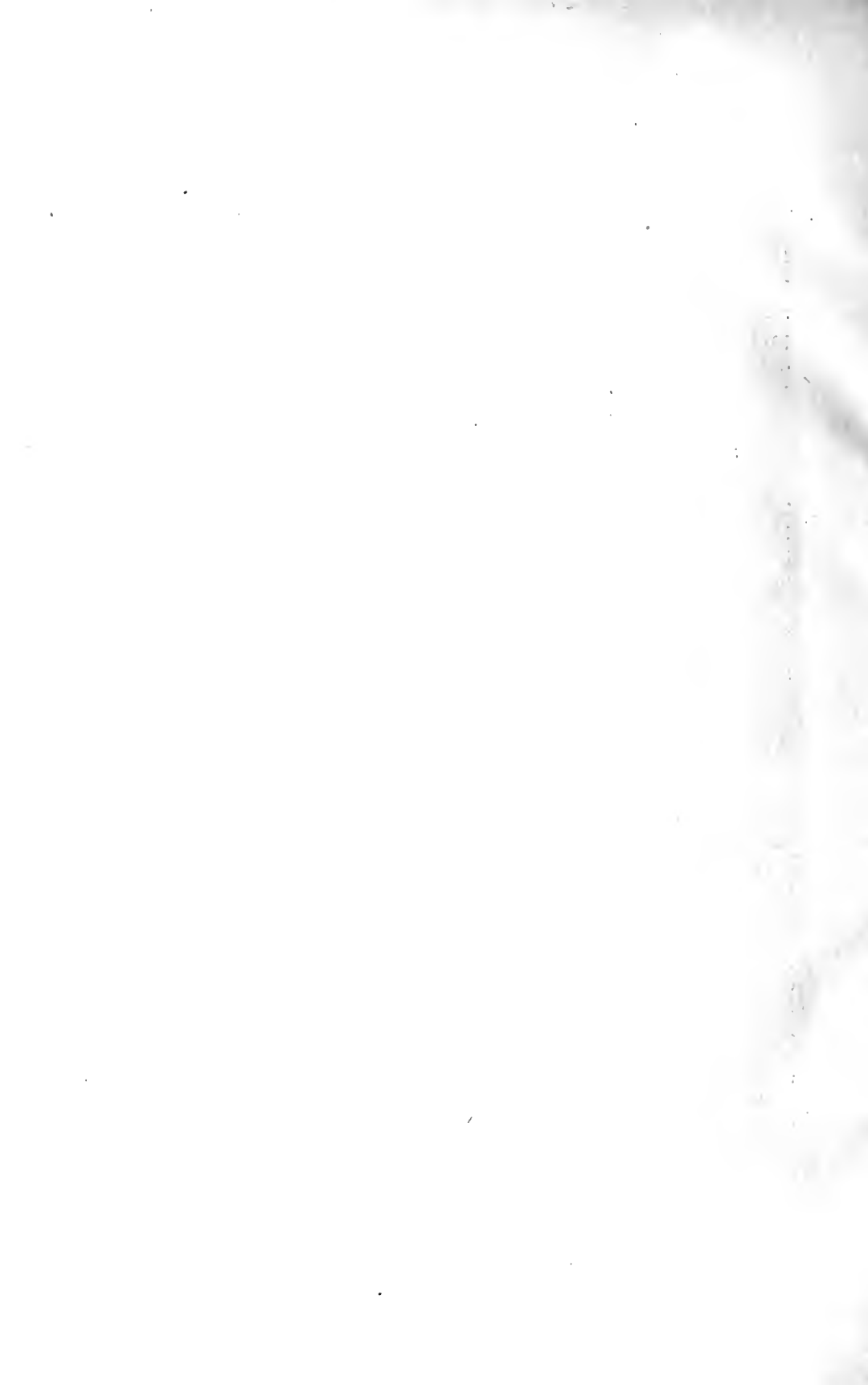
FIG. 3. Drawing showing the pulmonary artery and branches. The numbers designate the positions at which the emboli used in Protocol 1 lodged.

## PLATE 36.

FIG. 4. Kymograph record of blood pressure and respiration in Protocol 2. Time in minutes and seconds. Normal blood pressure 60 mm. of mercury. At the signals the clots of the animal's own blood were allowed to enter the circulation. Death was due to a large clot from the right iliac vein which completely blocked the pulmonary artery (Signal 2).

FIG. 5. Kymograph record of blood pressure and respiration. Time in minutes and seconds. Normal blood pressure 100 mm. of mercury. Each signal marks the passage of an embolus of a blood clot of the animal's own blood from either the left jugular or the right iliac veins. The clots were made as described in the text. Note the drops in the blood pressure after the entrance of each embolus. The break in the record covers a space of 6 minutes. At autopsy both branches of the pulmonary artery were found to be blocked with the clots formed in the veins.

FIG. 6. Kymograph record of blood pressure and respiration. Time in minutes and seconds. Normal blood pressure 125 mm. of mercury. The vagi were sectioned. The signals mark the time of the insertion of the paraffin emboli (4 by 0.5 cm.) into the right femoral vein. Note that the emboli cause the small drops in the blood pressure even after section of the vagi. Death was not produced until the pulmonary artery and right ventricle were blocked.





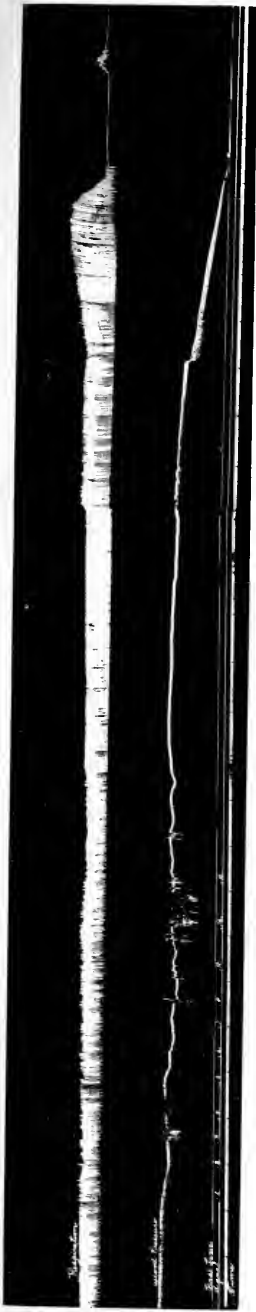


FIG. 1.

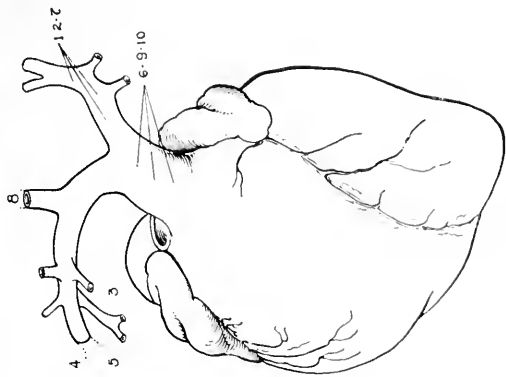


FIG. 3.

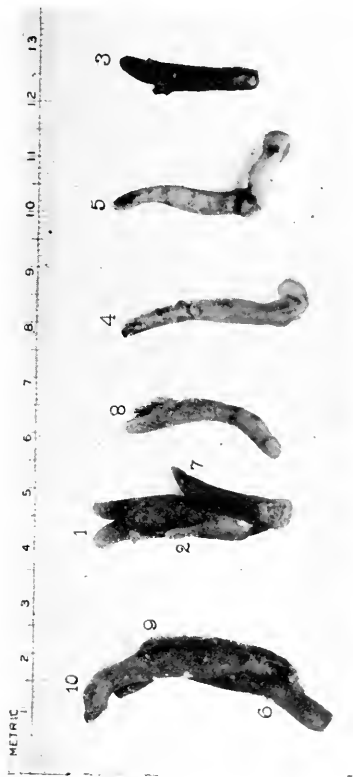


FIG. 2.

(Mann: Pulmonary embolism.)





FIG. 4.

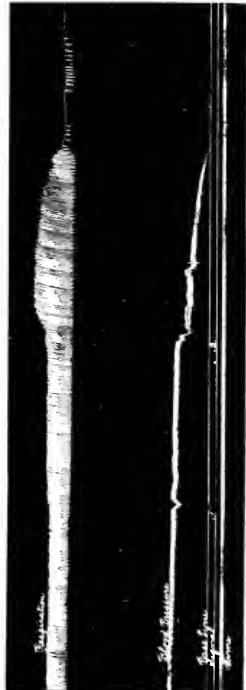


FIG 5.

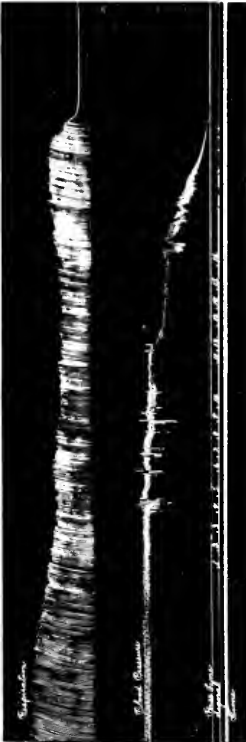


FIG. 6.



## THE VISCOUS METAMORPHOSIS OF THE BLOOD PLATELETS.

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PLATE 37.

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The phenomenon of agglutination and fusion of the blood platelets into glassy masses and strands, as observed in films of wet blood between a slide and cover-glass, was first described by Hayem (1) in 1878. Some years later, it was more exactly described by Bizzozzero (2) and named "viscous metamorphosis" by Eberth and Schimmelbusch (3). It occurs during the process of coagulation and is well marked at the time of the first appearance of the fibrin threads. It does not take place if sodium oxalate or certain other salts have been added to the blood during the bleeding in sufficient amount to prevent coagulation and if other conditions are favorable. Mosen (4) in 1893 was the first to show that platelets from such a non-coagulating blood, freed from blood elements by centrifuging, do not fuse with one another, but retain their separate identity. They appear when in suspension in isotonic salt solution and observed with medium powers between a cover-glass and slide, as refringent, glistening granules or round bodies of a diameter varying approximately from one-fourth to one-half that of a red blood corpuscle (Fig. 1); they are not clumped but uniformly distributed and remain so until they disintegrate. We have found, however, that such platelets normally have not lost the ability of metamorphosis, but that they undergo the characteristic changes within a few minutes if serum or certain more or less closely related substances are added to the suspension. As observed under the conditions just mentioned, the platelets begin their metamorphosis by aggregating and agglutinating in masses and strands which increase in size up to a certain point within a few minutes. Then within a few more minutes, the plate-

lets in these masses and strands lose their high refrangibility, seem to fuse one with another, and their separate identity becomes lost or obscure. The masses and strands assume thereby the appearance of a more or less homogeneous, glassy, refringent substance in which refringent granules are usually present. During this transformation or viscous metamorphosis some contraction of the volume of the masses and strands may occur and by this contraction in volume, bands and strings of hyaline material may be produced, which unite masses of platelets in the process of transformation or already transformed. The peripheral or marginal portions of the masses and strands are usually more hyaline than the central parts and the marginal outlines are finely wavy and denticulate.

This metamorphosis of the isolated platelets in the presence of serum has been briefly described in recent papers by Lee and Robertson (5) and by Minot and Lee (6) in which also some results of experiments with other substances are noted. The present paper is based on further experiments on this line in the endeavor to determine the nature of the substance in the serum which causes or permits the metamorphosis to take place.

It should be noted that this metamorphosis is to be distinguished from simple agglutination of platelets without fusion, which may be caused by certain substances. The microscopic appearances of a weak simple agglutination are shown in Fig. 2. Here the agglutinated platelets retain their separate identity.

Figs. 3, 4, and 5 show the appearances of suspensions of platelets similar to that shown in Fig. 1; the suspensions have undergone the viscous metamorphosis.

Lee and Robertson (5) have shown, and it may be emphasized here, that this phenomenon of agglutination and fusion of platelets associated with coagulation is to be distinguished from agglutination and lysis of platelets caused by antiplatelet serum.

The phenomenon seems to begin when the first signs of clotting are seen and is thus perhaps the first demonstrable sign of the clotting process. This has been our experience with not only normal blood, but blood with delayed coagulation time, as from cases of hemophilia, pneumonia, and jaundice, no matter whether observed in whole blood in glass tubes, or from cloudy plasma (plasma relatively rich

in platelets) obtained from centrifugalized whole blood in iced paraffined tubes. In the abnormally slow coagulating cloudy plasma one may observe that clotting begins near the edge of the tube and microscopic preparations from this portion of the plasma will show the fusion of the platelets, while material obtained at the same time from the still fluid portion will show the platelets free.

### *Method.*

Rabbit and human platelets were used. The blood was drawn from the rabbit's ventricle or from the veins of man by means of a needle and syringe. Before drawing the blood, a sufficient quantity of freshly prepared 1 per cent solution of sodium oxalate in 0.9 per cent sodium chloride solution was placed in the barrel of the syringe to constitute at least one part to nine parts of blood. Sometimes the needle and barrel of the syringe were coated with paraffin. In order to obtain platelets that are unaltered and suitable for studying the viscous metamorphosis, it is important that the needle enter the ventricle or the vein at the first thrust so as to avoid contamination with the tissue juice as much as possible, and that the blood should flow quickly and freely into the syringe and be rapidly mixed with the oxalate solution. If these conditions are not observed, the platelets are likely to undergo more or less fusion before isolation or to be otherwise altered so that they will not undergo the characteristic metamorphosis. Slight alteration in them, inappreciable in any change in microscopic appearance and often impossible to control, seems to render them incapable of undergoing the changes. Many times we have isolated platelets in apparently intact and unaltered condition which showed themselves but feebly active or inert.

On the other hand, platelets may be quite grossly altered without essentially affecting their activity as cytozyme (Bordet and Delange (7) ) or as thromboplastin (Howell (8) ). Even after they have undergone their metamorphosis we have found them capable of acting efficiently as factors in coagulation of the blood.

The platelets were separated from the other elements of the blood by centrifuging at low and high speeds, and were at least twice sus-

pended and thrown down in 0.9 per cent sodium chloride solution. Unaltered platelets, thus isolated and washed, form a white, semi-fluid, somewhat sticky sediment at the bottom of the centrifuge tube and they are readily miscible with salt solution by agitation with the pipette. If the sediment of the platelets forms an elastic coherent mass or masses, some metamorphosis has already occurred and such platelets are not suitable for use.

The isolated platelets in salt solution retain their ability to undergo the metamorphosis for a period varying from usually a few hours to rarely 2 or 3 days. This ability seemed to be retained longer at room temperature than in the ice box or incubator, and also if a very small amount of oxalate had been added to the salt solution.

The blood elements used in studying this phenomenon of agglutination and fusion of the blood platelets were usually obtained from rabbits. Most of the experiments were also done with human material. No differences in the results were noted when the platelets came from man or rabbit and the various other substances came from the same animal or a different animal of the same or different species. It was found rather more difficult to obtain suitable platelets from the arm veins of normal men than from the hearts of normal rabbits.

In testing the effect of the various fluids and solutions on the isolated platelets a thick suspension in 0.9 per cent sodium chloride solution was found best to use. This was prepared usually by mixing the sediment of platelets at the bottom of the centrifuge tube with three or four drops of the solution. It was found important that the platelets should be well mixed with this salt solution so as to be separated from one another and that they should be used in a very thick suspension as described. A very thin suspension of platelets was found undesirable for it seemed possible for the platelets to go through their metamorphosis in but tiny clumps or singly, so that the phenomenon was not clearly observable. Again, very compact masses of platelets were not used both on account of a possibility of an excess of platelets altering the results and also because the substance affecting the platelets may not penetrate to the center of the mass so that the metamorphosis will be observed only about the edge of the mass.



After a satisfactory suspension of blood platelets was obtained the action of various substances upon them was observed by mixing from one to three small drops of the materials in question with a very small drop (obtained from capillary glass tubing) of the very thick platelet suspension on a glass slide, covering with a cover-glass, and observing microscopically.

*Effect of Serum, Thrombin, Calcium, and Antithrombin and Thromboplastin.*

Serum was capable of producing well marked metamorphosis of the platelets usually in 2 to 7 minutes. Sometimes the change did not occur until after longer periods, up to 20 minutes or more. The time required varied both with different lots of platelets and with different sera. It was not clearly dependent on the age of the serum, although it may be said that sera a few hours old often seemed more effective than when very fresh, or sera 10 or more hours old.<sup>1</sup>

This power of serum was found present on some occasions in dilutions (made with 0.9 per cent salt solution) of 1:10, on other occasions in dilutions as high as 1:50. The greater the dilution the longer it took for the metamorphosis to occur. When calcium was added to the serum dilutions so that they contained 0.05 per cent calcium chloride it accelerated the metamorphosis and caused it to appear with those dilutions in which only slight agglutination had occurred. It is to be noted that with the higher dilutions that caused metamorphosis relatively fewer platelets seemed to be transformed than with the lower dilutions, suggesting a quantitative action of the substance that metamorphoses platelets.

Table I is a typical protocol showing the effect of dilution of serum with and without calcium.

With serum this phenomenon can be demonstrated macroscopically. If several small drops of a thick platelet suspension are added to 1 cc. of serum they will be at first evenly distributed through the serum giving it a cloudy appearance. When the metamorphosis has occurred they will be found clumped in the bottom of the tube and

<sup>1</sup> Rarely a serum produced no effect.

TABLE I.

Serum dilution with 0.9 per cent sodium chloride solution.	One drop of 0.9 per cent sodium chloride solution.		One drop of 0.5 per cent calcium chloride solution in 0.9 per cent sodium chloride solution.	
		<i>min.</i>		<i>min.</i>
0	F*	2	F	2
1 : 6	F	5	F	2
1 : 15	F	8	F	4
1 : 25	F	13	F	6
1 : 40	F	20	F	12
1 : 60	A	15	F	18
1 : 80	A?	20+	F	20
1 : 100	0		F	25
1 : 120	0		A	20
1 : 150	0		A?	25
1 : 200	0		0	

\* The figures in the table give the time in minutes for definite fusion (F) (viscous metamorphosis) or slight agglutination (A) of the platelets to take place under the given conditions.

upon shaking the tube the platelets will be seen to appear as small granular masses in a clear serum.

When platelets were treated with distilled water some portion of them went into solution and they lost their glistening appearance. Such a solution has definite thromboplastic (cytozymic) action in contrast to salt solution in which platelets have stood and been removed. The remaining shells of the platelets found after the platelets had been exposed to water were slightly clumped by serum, but a true metamorphosis did not take place.

Serum heated to 56°C. for half an hour or serum heated up to 60°C., at which temperatures thrombin is destroyed, caused no metamorphosis of platelets but did permit an agglutination of the platelets, and on some occasions seemed to allow a very slight lysis of the platelets. Platelets so treated, however, could still be metamorphosed by unheated serum, though possibly not so actively as when added directly to serum. This heated serum contained, as far as elements of coagulation are concerned, essentially only antithrombin and calcium.

From the above experiments we were inclined to believe that the viscous metamorphosis of platelets was due essentially to thrombin

aided by calcium. This, however, does not seem to be true, not only because of the results with "serozyme" (7), egg white, etc., described later, but also because repeatedly solutions of very active pure crystallized thrombin, prepared by Howell's method (9), did not produce metamorphosis of the platelets; although a slight slow clumping sometimes occurred there was never any fusion. If, however, calcium was added to the pure thrombin solution the platelets were often quite rapidly agglutinated into little ball-like masses but there was no metamorphosis.

Centrifuged, clear, oxalated plasma heated to 60°C. contains antithrombin but essentially no other coagulating element. Such antithrombin produced usually a very slight agglutination but no metamorphosis of the platelets. If, however, calcium was added, an agglutination, and possibly on some occasions, slight lysis occurred exactly as occurred but to a greater degree with the heated serum which contained the same two elements, antithrombin and calcium. When thrombin and antithrombin were mixed and allowed to stand 1 minute or 1 hour, the effect of the mixture on the platelets was the same as with thrombin or antithrombin alone. Antithrombin incubated with serum for a longer or shorter period did not have any definite effect on the ability of the serum to metamorphose platelets.

When pure thrombin and antithrombin from serum heated to 60°C. were mixed for 1 minute or 1 hour and calcium was added before or after the substances had remained in contact, the action of the mixture on the platelets was the same. Such a mixture produced a marked clumping of the platelets, faster and more marked than with thrombin and calcium or antithrombin and calcium. Occasionally this mixture also slowly caused a true metamorphosis of the platelets. Usually this was found not to be the case, but rather that besides the agglutination, a pseudo- or abortive metamorphosis occurred. This pseudo- or abortive metamorphosis consisted simply of a very marked agglutination with apparently a slight lysis of the platelets. On staining with Wright's blood stain the individual platelets in these masses were distinct and fairly sharply outlined and did not tend to form a homogeneous fused mass as was seen when true viscous metamorphosis occurred. Platelets so altered or platelets simply agglutinated by antithrombin and calcium or thrombin and

calcium were still capable of being metamorphosed with serum, but apparently not quite so effectively as unaltered platelets. This indicates that the process of agglutination of the platelets is to be distinguished from the viscous metamorphosis, though agglutination of some type is an integral part of the viscous phenomenon.

When mixtures of pure thrombin and calcium with antithrombin from oxalated plasma rather than from serum were mixed with platelets we never observed a true metamorphosis. Such a mixture did allow definite agglutination and at times a weak pseudo- or abortive metamorphosis.

Thromboplastin in various forms (fresh tissue juice, cephalin, or coagulen<sup>2</sup>) did not apparently influence the platelets either alone or when added in reasonable amount to any of the various substances or mixtures in the experiments outlined above or below.

*Effect of Serozyme, Oxalated Plasma Plus Thrombin, and Fibrinogen.*

Oxalated plasma whether dialyzed or not had, of course, no effect on the platelets, and when oxalate was added to any of the substances referred to above or below, it either prevented or weakened (if relatively little was added) their action on platelets. When the amount of calcium that clotted most quickly a given oxalated plasma was mixed with it and platelets were added at once to the mixture, which was then observed under the microscope, the platelets were seen to undergo their typical transformation. They began to do so as the first signs of coagulation appeared. In such a mixture, however, they completed their metamorphosis more slowly than under more favorable conditions. This we attributed to the presence of oxalate, for when a plasma had been dialyzed to remove the excess of oxalate, and calcium was then added to it with platelets, the latter elements underwent their metamorphosis more rapidly than when the control undialyzed oxalated plasma was used. This was true whether fresh plasma or dried plasma a year old, but freshly dissolved, was used.

The fluid resulting from the defibrination of a clot, formed with oxalated plasma and an optimal amount of calcium, is called by

<sup>2</sup> Commercial preparation made by the Society of Chemical Industry, Basle, Switzerland.

Bordet and Delange (7) "serozyme." When this is freshly prepared it often contains free thrombin. On standing some of the thrombin seems to combine with antithrombin and other portions of the thrombin become perhaps altered in some manner so that free thrombin cannot be demonstrated. A good serozyme, *i.e.*, one without free thrombin, as evidenced by its inability to clot fibrinogen in 24 hours, is to be obtained from a very clear rather than a cloudy plasma. Serozyme and cytozyme (tissue juice, thromboplastic substance) and calcium form thrombin in Bordet and Delange's terminology as is shown by the fact that after this mixture has stood for about 6 minutes it clots fibrinogen in 1 to 2 minutes, while suitable controls form no thrombin. Serozyme was found to be usually as active in causing the metamorphosis of platelets as serum, and its activity increased if a mere trace of calcium was added to it. It seemed to make little difference whether the serozyme contained free thrombin or not. Though rather definite in some instances but not in all, a serozyme containing free thrombin, prepared from cloudy plasma, seemed to be not quite so active in causing the platelet metamorphosis as a serozyme that had no free thrombin. This would suggest that the substance causing platelet metamorphosis had been partly used up in transforming the platelets in the cloudy plasma.

The following observations on the action of a mixture of Howell's pure thrombin and oxalated plasma were made. When these two substances, which form a clot in about 3 minutes, were mixed, and platelets added at once, the typical viscous metamorphosis of the platelets occurred usually in 2 to 3 minutes, provided apparently that the oxalated plasma was not oxalated to contain above about 0.1 per cent of oxalate. On some occasions, usually if the oxalation was excessive, only a rather marked agglutination or pseudometamorphosis of the platelets occurred about the fibrin strands, which suggests, as do the experiments with the mixture of thrombin, serum antithrombin, and calcium, that this phenomenon is an abortive type of true metamorphosis.

When thrombin and dialyzed oxalated plasma were used the platelets were always metamorphosed.

We may note that presumably all the substances mentioned before which produced this change in the platelets contained at least

traces of ionized calcium. Perhaps, with the rearrangement of the colloidal substances which occurs when thrombin clots the fibrinogen of the oxalated plasma, some calcium is temporarily set free. That some substance, as well as possibly calcium, is set free in this reaction which metamorphoses the platelets, is suggested by the above experiments, and that this substance then rapidly becomes relatively inactive is suggested by the following observations.

The fluid resulting from the complete clotting of oxalated plasma with thrombin seldom permitted the viscous metamorphosis of the platelets; sometimes a very slow and poor but true transformation occurred. This result was probably due to the oxalate, because the fluid resulting from thrombin and dialyzed plasma did permit, though always slowly (20 minutes) the transformation of the platelets. Such delayed reactions are in contrast to the relatively rapid effect on platelets during the clotting of plasma with thrombin. If, however, calcium was added to either of the fluids resulting from clotting dialyzed or undialyzed oxalated plasma with thrombin, they then not only always permitted a metamorphosis of the platelets, but also a rapid one (5 minutes). This may have been partially due to the effect of calcium on the oxalate, besides its evident accelerating action, as well as perhaps to some change that occurred in these fluids when the calcium acted on the prothrombin present in them. That these fluids contained prothrombin is evident, because this substance was in the oxalated plasma and there had been no calcium added to transform it to thrombin.

Magnesium sulfate plasma and thrombin acted essentially like oxalated plasma and thrombin.

Solutions of fibrinogen, prepared according to a modification of Hammarsten's method, were found to have no effect on the platelets. These fibrinogen solutions unfortunately were not absolutely free of prothrombin, but contained enough to clot in 3 hours with the optimal amount of calcium. The results of experiments with thrombin and this fibrinogen were essentially like those with thrombin and dialyzed plasma, except that the metamorphosis of the platelets was never marked even when calcium was added to the serum from the coagulation of this fibrinogen by thrombin. It would seem that, during the process of clotting fibrinogen there occurred, with the

rearrangement of the colloidal particles, a setting free of some substance allied to serozyme which was very active on the platelets, and, with the completion of such coagulation, relatively little substance was left free, though addition of calcium, perhaps by its property of aggregating colloidal particles, would permit the substance causing platelet metamorphosis to become more active.

*Absorption from Serum of the Substance Causing Metamorphosis.*

Experiments were conducted to determine whether one could absorb from serum the substance that transforms platelets. Barium sulfate absorbs serozyme-like substances as shown by Bordet and Delange and Lee and Vincent (10), but it does not seem to affect formed thrombin in Bordet and Delange's sense. We found that when 1 cc. of serum was treated with 0.4 cc. of washed barium sulfate for 2 hours and then centrifuged clear, that the serum had lost its power of metamorphosing platelets even after calcium was added.

The effects of adding large amounts of cephalin to serum were next studied. A relatively large amount of cephalin in solid form was added to serum so that some of it dissolved in the serum. When this mixture was then centrifuged, so that the resulting fluid was clear, it was found to have lost its ability to metamorphose platelets, though sera so treated were able to clot fibrinogen. When calcium was added to such serum it did not restore the ability to metamorphose platelets. This effect of cephalin was obtained in but a third of the numerous times it was tried on 6 different days. It seemed that if the sera could be centrifuged so that they were clear after treatment with cephalin, this substance was much more apt to be effective than if the centrifuged sera remained at all cloudy. Sera, when somewhat diluted with salt solution to which thick suspensions of platelets were added and then centrifuged clear after the platelets were fully agglutinated and fused, sometimes lost their ability to further metamorphose platelets, as was the case with sera treated with cephalin. Undiluted sera could not be affected in this way, probably because too few platelets were added. Similar results were obtained upon treating sera with cholesterolized syphilitic antigen as with cephalin and platelets. We have found no explanation for these inconstant results.

It was thought that the power of syphilitic serum to metamorphose platelets might be more easily exhausted with syphilitic antigen than normal serum, in view of the inhibitory effect of syphilitic serum in Bordet and Delange's method of forming thrombin as shown by Hirschfeld and Klinger (11). No such difference, however, could be detected.

Starches of various kinds, agar, gelatin, gold chloride, cholesterol, and chloroform in various strengths, and various other substances did not, after being mixed with serum for 2 hours, alter its ability to produce this metamorphosis. Neither did these substances alone metamorphose the platelets.

*Ability of Serum Globulin and Egg White to Metamorphose Platelets.*

In order to determine whether the power of serum to metamorphose platelets resided in the globulin or albumin fraction, the following experiment was made. Serum was dialyzed against distilled water. When a small amount of precipitate had appeared at the bottom of the dialysis sac, the serum was still active. Later, when a considerable amount of precipitate occurred in the dialysis sac, the fluid with or without calcium was inactive on the platelets. The dialysate was also inactive. The precipitated globulin, when redissolved in salt solution with the aid of a trace of dilute alkali, which of itself did not affect the platelets, caused a feeble metamorphosis of the platelets. However, when calcium was added to this solution a definite active metamorphosis of the platelets was produced with it.

Egg white alone or shaken with isotonic salt solution had a marked effect on the platelets. It caused them to be rapidly metamorphosed and often seemed to act more efficiently than serum. This result suggested that egg white contains a serozyme-like element. Experiments showed this to be the case, for it was found that it could take the place of serozyme in the formation of thrombin by the method of Bordet and Delange. Hirschfeld and Klinger (12) have recently also detected a serozyme-like action of egg white. Egg white alone apparently does not clot fibrinogen and therefore does not contain formed thrombin.



Numerous pure proteins,<sup>3</sup> lactalbumin, gliadin, legumin, vignin, edestin, and cottonseed globulin, and also milk with or without calcium had no effect on the platelets.

*Metamorphosis of Platelets from Abnormal Bloods.*

The platelets from cases of hemophilia, pneumonia, purpura hæmorrhagica, purpura rheumatica, and obstructive jaundice were transformed by either their serum or normal serum in the same way and essentially in the same time as normal platelets. In this connection it is to be noted that hemophilic platelets are, however, defective in their cytozymic action and that they do not begin to fuse until the blood shows visible signs of clotting, as has been shown by Minot and Lee (6). On this account the metamorphosis of hemophilic platelets and normal platelets was studied with those substances causing metamorphosis of normal platelets, derived from normal and hemophilic blood, but no real differences in the character or rate of metamorphosis could be detected.

SUMMARY AND CONCLUSIONS.

From the experiments above described it is evident that the viscous metamorphosis of the blood platelets in shed blood is intimately associated with the early stages of coagulation and that the presence of calcium is a very important element, though perhaps not absolutely necessary. No constant differences in this phenomenon could be detected with platelets or coagulating elements from the blood of normal or diseased individuals or from man or rabbits.

All the substances, with the exception of egg white, causing this phenomenon were derived from the blood either during the alteration of fibrinogen or after it had been acted on by thrombin. It seems that the substances or mixtures capable of producing the metamorphosis are especially those associated with the early stages of coagulation or capable in the test-tube of forming or liberating that active coagulating element known as thrombin.

The substance in serum that is capable of metamorphosing platelets seems to be attached to the globulin fraction rather than the

<sup>3</sup> These proteins were kindly furnished by Dr. W. Denis.

albumin fraction and is destroyed by heat at temperatures which destroy prothrombin, thrombin, and serozyme, and precipitate fibrinogen.

The reaction is not caused by pure thrombin or a mixture of pure thrombin and calcium, though substances causing the metamorphosis are intimately related to thrombin. The metamorphosis seems to be caused by serozyme-like substances as shown both by the fact that barium sulfate absorbs the power of serum to cause the reaction and that a serozyme-like substance is probably to be recognized in all the substances or mixtures, including egg white, causing this change in the platelets, except perhaps when thrombin and fibrinogen are reacting. It is to be noted that in serozyme are contained antithrombin, calcium, and potential thrombin, and that a combination of these isolated factors mixed together occasionally allowed the viscous metamorphosis to occur and not infrequently an abortive metamorphosis.

The pseudo- or abortive metamorphosis caused by the mixture of pure thrombin, antithrombin, and calcium may be interpreted on the supposition of a close approximation but not a real reproduction of the colloidal state known as serozyme. That such a reaction is related to real viscous metamorphosis is suggested, because it sometimes occurred with the above mixture and when thrombin and heavily oxalated plasmas were reacting, rather than a real metamorphosis.

Simple agglutination of the platelets may occur independently of a viscous metamorphosis, though an agglutination of platelets is to be considered an integral part of the viscous metamorphosis phenomenon.

The inconstant results seen with the cephalin-treated sera are probably due to the fact that exactly the same mixtures were not obtained. The results with cephalin-soaked and platelet-soaked sera and with dilutions of sera suggest that the reaction of viscous metamorphosis of the platelets is quantitative.

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EXPLANATION OF PLATE 37.<sup>4</sup>

FIG. 1. Suspension of blood platelets in isotonic salt solution.  $\times 300$ .

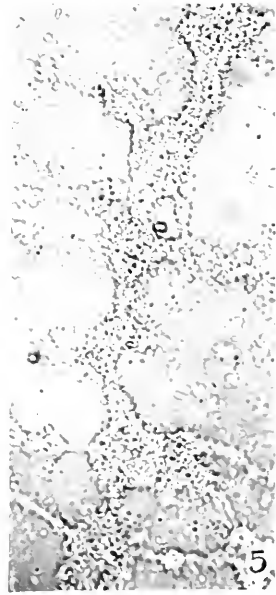
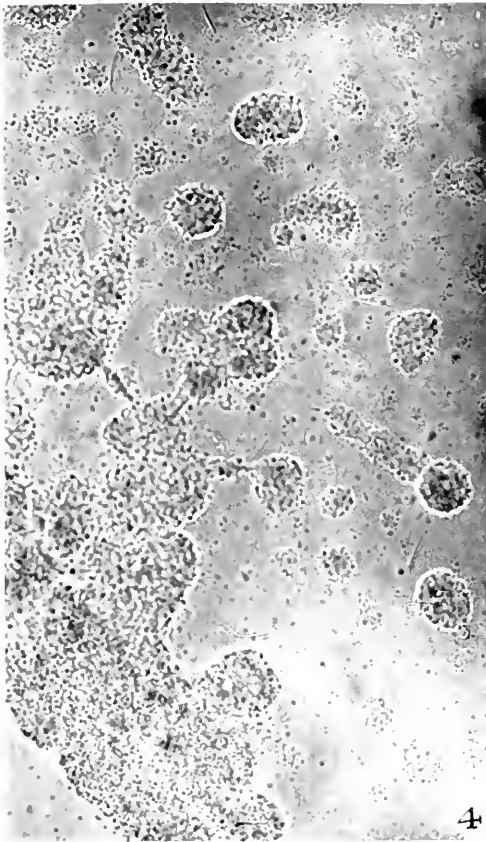
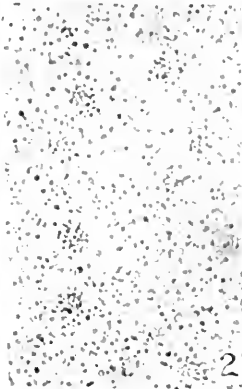
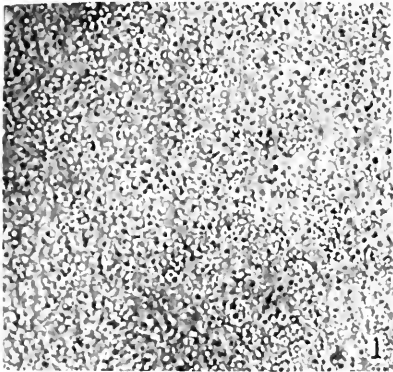
FIG. 2. Simple, weak agglutination of platelets. There are fewer platelets in this preparation than in those shown in the other figures.  $\times 300$ .

FIGS. 3, 4, and 5. Viscous metamorphosis of platelets. Essentially the same number of platelets was in these preparations as in the preparation shown in Fig. 1.  $\times 300$ .

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<sup>4</sup> The photographs were made by Mr. L. S. Brown.





(Wright and Minot: Viscous metamorphosis of blood platelets.)



## STUDIES ON BACTERIAL ANAPHYLAXIS AND INFECTION.

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PLATES 38 AND 39.

(Received for publication, April 19, 1917.)

Since anaphylaxis has now been clearly recognized as a phenomenon which depends upon an antigen-antibody reaction, it follows naturally that the fundamental principles underlying these manifestations should apply, subject perhaps to individual modifying factors, to all substances which on injection into the animal body induce specific antibody formation. By far the greater volume of work on anaphylaxis has been carried out with antigens consisting of animal sera, egg white, and other freely suspended proteins not enclosed in cells. And in the case of such serum anaphylaxis it has been possible to go far in the analysis of the mechanism of the phenomenon and its application to such practical problems as serum sickness and other clinical conditions referable to abnormal protein metabolism.

There has been relatively less progress, however, in the study of the hypersusceptibility incident to the treatment of animals with cellular antigens; that is, with bacteria, erythrocytes, and other cells. And yet these phenomena in the case of bacteria may reasonably be assumed to play an important part in the symptomatology and pathology of infectious diseases where there is a continuous liberation of fluctuating amounts of specific antigen in a body at first perhaps unsensitized, but later increasingly hypersusceptible, and perhaps repeatedly sensitized and desensitized during the course of the disease. Of these conditions we know very little, and correspondingly much speculation and reasoning from the analogies of serum anaphylaxis have been the bases of many inferences and theoretical suggestions.

It is plain that when the antigen employed is a cellular one we are necessarily dealing with a phenomenon consisting of two phases. As integral parts of compact structures, the cell constituents cannot react as antigen with the cells and fluids of the invaded body until they have been liberated by cell disintegration. After that the process may be, and probably is, entirely similar to serum anaphylaxis. But before this state of affairs can be brought about it must be assumed that some preliminary reaction occurs in which the bacterial protein is set free from the cell. As to the nature of this reaction we have no certain knowledge. We cannot be sure at present, at least in our opinion, whether such a reaction is or is not accompanied by the direct formation of toxic cleavage products within the circulation such as those produced *in vitro* by Vaughan,<sup>1</sup> Friedberger,<sup>2</sup> and others, though this seems likely. Moreover, we do not know to what degree and under what circumstances such a process may be interfered with by intercurrent phagocytosis, and perhaps by bacterial agglutination and accumulation in the viscera, as demonstrated by Bull.<sup>3</sup>

The practical bearing of bacterial anaphylaxis on the course and symptomatology of infectious disease is of necessity very great. It has been suggested that the incubation time of infections may be in part dependent upon the gradual development of the specific sensitization of the tissues as antibodies begin to appear. The evidence adduced by the study of the isolated uterus, intestinal musculature, the perfused heart and lungs, and such experiments as those of Longcope<sup>4</sup> on the kidneys of rabbits points to the possibility that many of the insults offered various tissues, formerly attributed to specific preformed bacterial poisons, may be anaphylactic in nature. It is not impossible that a process of alternate sensitization and desensitization, either interrupted or continuous and gradual, may represent the dominating mechanism of injury in bacterial disease.

<sup>1</sup> Vaughan, V. C., Vaughan, V. C., Jr., and Vaughan, J. W., Protein split products in relation to immunity and disease, Philadelphia and New York, 1913.

<sup>2</sup> Friedberger, E., Series of papers in *Z. Immunitätsforsch., Orig.*, 1910 to 1913.

<sup>3</sup> Bull, C. G., *J. Exp. Med.*, 1915, xxii, 475.

<sup>4</sup> Longcope, W. T., *J. Exp. Med.*, 1913, xviii, 678.



We do not yet understand clearly the relation of the circulating antibodies to such processes. Do they aid in the intravascular mechanism for the liberation of toxic substances as suggested by Pfeiffer, Radziewsky, Wolff-Eisner, and more recently by Vaughan, Friedberger, and others? Or do they both hasten the removal of antigen by bactericidal and opsonic action and perhaps even protect the sensitized tissues by combining with the circulating antigen and diverting it from the sessile antibodies of the sensitized tissues? It would be interesting and important to know, moreover, whether or not there is a true bacterial hypersusceptibility in the sense that an animal treated with dead bacteria or bacterial products (as in vaccination) might pass through a preliminary period during which susceptibility to invasion by the living organisms or injury by their body constituents is increased.

All these problems await an analysis of bacterial anaphylaxis as thorough as that which has been made with antigens of non-cellular nature.

In order to approach the problem properly it is desirable to review briefly what has been done and how much of it may be accepted as needing no further inquiry.

The first investigators who systematically studied serum anaphylaxis naturally turned their attention, also, to anaphylaxis produced with bacterial protein. Thus Rosenau and Anderson<sup>5</sup> obtained definite results with extracts of various bacteria, using chiefly colon bacilli, extracts of yeast, of *B. subtilis*, and of typhoid bacilli. They injected relatively large quantities of the extracts subcutaneously into guinea pigs and, after periods of from 19 to 35 days, reinjected intraperitoneally or subcutaneously. They obtained reactions which they characterized as "mild," "marked," or "severe," but rarely observed acute anaphylactic death. Subsequently a large number of investigators turned their attention to the same problem and more or less divergent results were reported. The work was done chiefly by Kraus and Doerr,<sup>6</sup> Kraus and Amiradžibi,<sup>7</sup> Holobut,<sup>8</sup> Yamanouchi,<sup>9</sup> Delanoë,<sup>10</sup> Ascoli,<sup>11</sup> and Friedberger and his associates. It is hardly necessary

<sup>5</sup> Rosenau, M. J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H.*, 36, 1907.

<sup>6</sup> Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1908, xxi, 1008.

<sup>7</sup> Kraus, R., and Amiradžibi, F. S., *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 607.

<sup>8</sup> Holobut, T., *Z. Immunitätsforsch., Orig.*, 1909, iii, 639.

<sup>9</sup> Yamanouchi, T., *Compt. rend. Soc. biol.*, 1909, lxvi, 531.

<sup>10</sup> Delanoë, M. P., *Compt. rend. Soc. biol.*, 1909, lxvi, 207, 252, 348, 389.

<sup>11</sup> Ascoli, M., *Compt. rend. Soc. biol.*, 1908, lxv, 611.

to go into a detailed historical review of the literature since the difficulties encountered, and the general conclusions drawn, may be summarized very simply. Most of the work done was carried out with definite prejudice in favor of complete analogy between serum hypersensitiveness and bacterial anaphylaxis. Kraus and Doerr, as well as Holobut and others, claimed that sensitization could be accomplished with regularity only if small doses of bacteria, *i.e.*, one one-hundredth of a loopful, were given daily for 10 days and reinjection was practiced about 3 weeks after the last injection with a relatively large amount washed up with and partly extracted in slightly alkalized salt solution. It was found by a number of observers that the results were not uniform with different species of bacteria. It apparently has been very difficult, according to Müller, to sensitize with streptococci perhaps, as Müller suggests, because of the relative insolubility of these bacteria. Moreover, acute symptoms on reinjection in bacterial anaphylaxis have not been obtained with regularity and have been comparable to the acute shock in serum anaphylaxis in the smaller number of cases only.

Delanoë has denied the strict specificity of bacterial anaphylaxis, claiming that animals sensitized with typhoid bacilli reacted distinctly when reinjected with colon and paratyphoid A and B bacilli. This, however, may be true and yet fail to contradict the essential specificity of bacterial anaphylaxis as asserted by most investigators, since a group reaction might easily have accounted for his results, together with the toxic reaction so often noticed on first injection of any bacterial protein into guinea pigs.

Passive sensitization against bacterial protein was first reported by Kraus and Doerr and was further worked upon by Kraus and Amiradžibi. They injected guinea pigs with the serum of immune guinea pigs and rabbits, and 24 hours later injected the antigen intravenously or intraperitoneally, obtaining acute shock which sometimes killed the animals. Delanoë reported similar experiments and Yamanouchi and Ascoli suggested the utilization of passive anaphylaxis for the diagnosis of tuberculosis and typhoid fever.

Kraus and Amiradžibi also published experiments in which they obtained shock in guinea pigs into which they had injected typhoid bacilli mixed with the immune rabbit serum just before injection. These experiments are cited throughout the literature as pointing to the intravascular mechanism of bacterial anaphylaxis. Whatever may be our opinion concerning this question, and we will further discuss this in another place, the experiments of Kraus and Amiradžibi are inconclusive since they entirely disregard the not infrequent toxicity of normal and immune rabbit serum for guinea pigs, a disturbing factor which we encountered very early in our own work. In their protocols they record positive results only when the dose of serum was equal to or exceeded 0.5 cc. of rabbit serum and they confirmed the suspicion that serum toxicity was the true reason for the observed shock by stating that the active rabbit serum had lost its ability to give reactions when injected with bacteria after having been pre-

served for a month, a fact which our own investigations and the work of others have shown to be the case with sera toxic for animals of another species. The experiments of Kraus and Amiradžibi therefore, may be disregarded as having any bearing on the intravascular nature of bacterial anaphylaxis.

Nevertheless, from the work of other observers (Friedberger and Mita, Delanoë, and others) it seems beyond question that, just as one would expect, passive sensitization of guinea pigs with the sera of animals sensitized or immune to bacteria is possible. There is some discrepancy, however, between the results obtained by us and those reported by the observers mentioned in regard to the time necessary between injection of the antiserum and the development of the hypersensitiveness. This is a matter concerning which we will have more to say below.

As far, then, as reaction with the dissolved bacterial antigen is concerned there seems to be a complete analogy between serum and bacterial anaphylaxis except in one important and perhaps fundamental point, and this is the fact of primary toxicity of the bacterial protein. For, while the first inoculation of such antigens as horse serum, sheep serum, egg white, etc., produces no symptoms of injury in the injected animal, typhoid protein and that of many other bacteria if given in sufficient amounts lead even at first injection to severe illness and rapid or delayed death according to the variety of bacteria and the amount injected.

Indeed it is an observation recorded by Friedberger and Mita, by Seitz,<sup>12</sup> by Müller,<sup>13</sup> and by Doerr,<sup>14</sup> as well as occasionally made in our own experiments, that typical anaphylactic symptoms may follow immediately upon first injection of bacteria and bacterial extracts, if the amount given is sufficient. Müller particularly, has subjected this occurrence to systematic study. He finds that the chief difference between the reaction of the sensitized and the unsensitized guinea pig to intravenous injection of bacterial protein consists in the facts that the sensitized animal will show acute reaction to doses smaller than those necessary to produce the same result in the normal controls and that these smaller doses, which produce acute anaphylactic death in the sensitized, will produce a

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<sup>12</sup> Seitz, A., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 91.

<sup>13</sup> Müller, P. T., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 426.

<sup>14</sup> Doerr, R., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, ii, 1098.

more protracted illness and often death after the lapse of hours or days in the unsensitized. Müller interprets his work entirely in the sense of Friedberger's anaphylatoxin theory, in that he believes acute death to be due to the formation in the blood stream of a poison resulting from the reaction between plasma and bacteria, a process which is therefore more rapid in the sensitized animals in whose blood stream antibodies are present in relatively higher concentration.

These considerations bring the problem of bacterial anaphylaxis into intimate relationship with questions concerning the nature of the toxic properties of such microorganisms as the typhoid, paratyphoid, and plague bacilli, the cholera spirillum, and other bacteria, which unlike diphtheria and tetanus, do not secrete true toxins or exotoxins. Friedberger's theory would both explain anaphylactic poisoning with bacterial antigens and relegate to the chapter of discarded theories the older endotoxin conception of Pfeiffer which assumed the existence of specific preformed intracellular poisons liberated mechanically by disintegration of the cell body.

As a matter of fact, the entire problem of endotoxins is one which calls for reexamination in that the knowledge gained of recent years has opened a number of alternative explanations for the primary toxicity of such bacteria as the typhoid bacillus. Briefly summarized they are: (1) The actual intracellular existence of specific endotoxins in the sense of Pfeiffer<sup>15</sup> (toxalbumin). (2) The production of toxic split products in the animal body from the bacterial protein by proteolytic cleavage brought about by non-specific serum protease (Jobling and Petersen<sup>16</sup>) or by the cooperation of antibody and alexin (Friedberger). (3) The absorption of antienzymes by the bacteria with consequent activation of the serum protease which then splits off toxic substances from the plasma protein (Jobling and Petersen<sup>17</sup>). (4) The presence of non-specific toxic substances in the bacterial cell body, of the nature of peptones, primary and secondary albumoses, etc., which are liberated by lysis from the bacterial cell after cell death. This conception would differ from that of Pfeiffer in that the intracellular substances are conceived as in no sense specific toxic proteins, but rather entirely non-specific constituents repre-

<sup>15</sup> Pfeiffer, R., *Z. Hyg.*, 1892, xi, 393.

<sup>16</sup> Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xx, 452.

<sup>17</sup> Jobling and Petersen, *J. Exp. Med.*, 1914, xx, 321.

sending the type of poisons conceived as proteolytically produced from the antigen by Vaughan and others. This last view, though hitherto not particularly considered, should nevertheless in our opinion be regarded as at least a possible explanation for a part of the toxic manifestations resulting from the injection of bacteria of this class. Moreover, such a possibility is suggested by the fact that bacterial protein is relatively poor in antigenic properties. Doerr,<sup>18</sup> also, has considered this in stating that he believed the difficulty of producing anaphylaxis with bacteria was in part due to the fact that their body substances were relatively poor in coagulable (antigenic) proteins. We have not been able as yet to study the matter extensively, but we have carried out a few experiments, as follows:

Typhoid bacilli from twenty-four agar cultures were weighed as a moist mass, ground up with salt, and then taken up in distilled water to isotonicity. After the addition of 0.2 cc. of  $\frac{N}{1}$  sodium hydroxide to 100 cc. the suspension was heated to 60°C. for 30 minutes to prevent autolysis, and was then shaken for 4 to 5 hours with a motor, at room temperature. After filtration through a Berkefeld candle the clear solution gave a definite cloud after boiling and adding acetic acid. The filtrate was treated with heat and acid to remove coagulable protein. On the advice of Professor Gies the suspension was first brought to a boil and then small amounts of acid were added to prevent possible hydrolysis which might have occurred had the acid been added first. The filtrate from this was then half saturated with ammonium sulfate. Again a definite cloud was obtained, and when this was filtered clear, a second turbidity could be produced by complete saturation with the sulfate.

Although we have not yet obtained toxic reactions with these substances after isolation, perhaps because of the difficulty of obtaining them in sufficient amount, the presence of albumoses, substances which have often been found to possess primary toxicity for animals, suggests the possibility that their existence in the bacterial body might indeed contribute to the injury done by injections of bacteria. We found albumoses in extracts of typhoid bacilli not

<sup>18</sup> Doerr,<sup>14</sup> p. 1100.

only when the bacteria were grown on the ordinary peptone media but also on agar made without peptone to which nutrose or sodium caseinate has been added as an enriching substance.

Moreover, we have also had the experience with the typhoid bacillus which is referred to repeatedly by Vaughan when dealing with the colon bacillus; namely, that bacterial suspensions subjected to boiling are quite as toxic and often more so than are the unheated and living suspensions.

Thus, if three guinea pigs are injected with equal amounts of typhoid suspension, one with the living bacteria, the second with bacteria heated to 60°C. for 15 minutes, and the third with bacteria boiled for 5 minutes, the guinea pig receiving the boiled bacilli will often be the first one to grow sick and may die several hours before the others.

This may mean, of course, as Vaughan suggests, that the heated protein is more promptly split by the ferments of the body. It also suggests, however, that in addition to this the heating has left unchanged non-coagulable toxic constituents of the cell.

#### *Passive Sensitization of Guinea Pigs to Bacteria.*

Since in this work we were concerned primarily with the fundamental problems referred to in our introduction, we neglected for the present the purely technical difficulties connected with active sensitization and employed entirely the method of passive sensitization in which a more immediate and direct control over the relationship between the development of hypersensitiveness and the concentration of circulating antibodies could be exercised.

The experiments described in the following paragraphs illustrate the passive sensitization of guinea pigs by intraperitoneal injection with antityphoid serum. They show that unlike Kraus and his collaborators, we never succeeded in finding the animals sensitized in less than 3 to 5 days, the highest degree of sensitization being developed in about a week. This was true both when the whole animal was tested and when the reactions were obtained with the isolated uteri.

*Passive Sensitization to Typhoid Protein.* (a) *Experiment 1.*—Apr. 8, 1916. Guinea Pigs 1 to 5 inclusive are injected intraperitoneally with 1 cc. of Anti-typhoid Serum W which agglutinates 1:10,000.

Apr. 10. Guinea Pig 5, weight 220 gm., is injected intravenously with 2 cc. of typhoid extract. No immediate symptoms.

Agglutination titer of this animal 1:80++  
1:160+

Apr. 12. Guinea Pig 4, weight 190 gm., injected intravenously with 2 cc. of typhoid extract. Typical symptoms; falls to side; characteristic breathing; definite but not fatal shock. As well as control in 8 minutes. Control, weight 195 gm., shows no immediate symptoms.

Nos. 2 and 4 titrated on this day show agglutination up to 1:160.

(b) *Experiment 2.*—Oct. 18, 1916. A series of guinea pigs, weight 220 to 255 gm., is intraperitoneally injected with 2 cc. of antityphoid rabbit serum with a titer of 1:4,000.

Oct. 20. These guinea pigs show a titer of 1:100 to 1:200.

Oct. 23. Agglutinins in guinea pigs now about 1:100.

Oct. 24. Titer diminishing to 1:50 in most of them, others partially agglutinated 1:100. On this day one guinea pig is injected intravenously with 1 cc. of typhoid extract. No sign of anaphylaxis. This animal, however, died over night, whereas the unsensitized control of the same weight remained alive.

Oct. 25. Typical shock obtained in two of the guinea pigs; no immediate symptoms in controls on injection of 1 cc. of extract.

It would not aid much in throwing light on the subject of our paper were we to multiply experiments like the foregoing. We may summarize by stating that, after injecting intraperitoneally anti-typhoid serum, and waiting a period of 3 to 5 days, at a time when agglutinins are still present to a considerable degree in the circulation of the guinea pigs, definite shock can be observed on injection of antigen. Earlier than this, however, in spite of a higher concentration of antibodies in the blood, no definite or severe acute shock could be elicited. It has seemed that to obtain very severe symptoms an interval of considerably longer than 1 day is necessary when an intraperitoneal first injection is practiced.

The following experiment, one of several similar ones, shows that when relatively large amounts of antigen are used the differences between normal and sensitized guinea pigs are essentially quantitative and not qualitative:

*Experiment 3.*—Jan. 16, 1917. Guinea Pigs 6 and 7 are intraperitoneally sensitized with 2 cc. of antityphoid serum.

Jan. 19. The following injections are made.

Guinea Pig 6, weight 170 gm., receives intravenously 5 cc. of typhoid extract. Immediate shock; death in 8 to 10 minutes. Control, weight 200 gm., receives 5.5 cc. of same antigen; quite sick, coughs, scratches nose, breathes irregularly. Gradual recovery.

Guinea Pig 7 receives 4 cc. of antigen. Immediate difficult breathing, coughing, very sick. Died after 3 to 4 hours. Control, same weight, receives 4 cc. of antigen. Difficult breathing. Same type of illness as No. 7 but less severe. Both controls dead next morning. No. 7 dies in afternoon.

Although it has been an irregular observation, the immediate development of anaphylactic symptoms in normal guinea pigs injected with large amounts of typhoid antigen has occurred with sufficient frequency to force itself upon our consideration. A typical instance of this is the following:

*Experiment 4.*—Feb. 20, 1917. A normal guinea pig, weight 175 gm., received 3 cc. of typhoid extract intravenously at 12 noon. Immediate coughing, respiratory distress, staggering. Temporary improvement; died at 4 p.m.

This observation we think has important theoretical bearing when correlated with later experiments on the isolated uterus. In this place we wish merely to record it for future reference. It is interesting to note in this connection that, although the sensitized animals show a much greater tendency to immediate reaction, unless the shock is fatal, they rapidly improve and approach the condition of slower intoxication, ordinarily found in the normal animals similarly treated. The following experiment illustrates these conditions:

*Experiment 5.*—Feb. 28, 1917. Guinea Pig 8, weight 155 gm., sensitized intraperitoneally with 2 cc. of antityphoid serum on Feb. 24, was injected intravenously with 2 cc. of typhoid extract. At the same time a control normal animal, weight 135 gm., was similarly injected. The sensitized animal showed immediate symptoms; coughing, difficult respiration. The control showed no immediate effects. After 10 minutes the sensitized animal had improved and both were alike and growing progressively sicker. Both died on the same afternoon.

Although the preceding experiments seem to furnish sufficient evidence that the dominating mechanism of acute shock in bacterial anaphylaxis is not one of intravascular antigen-antibody reaction, we nevertheless thought it wise to repeat the experiments of Kraus



and Amiradžibi who obtained shock, as stated before, when they injected simultaneously bacteria and antiserum. This we did for the sake of completeness although we feel sure that Kraus and Amiradžibi's results were referable to the intrinsic toxicity of their rabbit serum for guinea pigs.

In the experiment which follows, one of several similar ones, antigen injections were made into guinea pigs passively sensitized, into normal animals, and into normal ones that had received intravenously 0.5 cc. of antiserum about an hour before inoculation with the antigen. One example of such an experiment follows.

*Experiment 6.*—Apr. 9, 1917. Guinea pig, weight 199 gm., passively sensitized. Agglutination titer on Mar. 30, 1:80. 1.45 p.m. Receives intravenously 2.5 cc. of typhoid antigen. Very sick immediately; breathes with difficulty and irregularly for 5 minutes, then better. 9 p.m. Died.

10.45 a.m. Normal guinea pig, weight 200 gm., receives intravenously 0.5 cc. of typhoid antiserum. 1.55 p.m. Inoculated intravenously with 2.5 cc. of antigen. Not quite as sick as the preceding animal; illness develops more gradually but this animal does not recover from immediate symptoms as rapidly as the other. Dies next afternoon at 2.45 p.m.; *i.e.*, lives 6 hours longer than preceding guinea pig.

Normal guinea pig, weight 195 gm., inoculated with 2.5 cc. of antigen. 2.30 p.m. Very slightly sick, almost not at all; gradually sick. Very sick in p.m., but recovers and remains alive.

It would be of relatively little value for the purposes of this paper were we to protocol other experiments similar to or representing variations of the preceding. The results were by no means regular but, taken together, they pointed to certain fundamental conditions which are of considerable interest in connection with our later work.

We may summarize these facts by saying that: (1) Acute shock with convulsions and death is obtained more frequently and with lower dosage in sensitized than in normal guinea pigs. (2) Acute shock with death cannot be obtained with regularity in sensitized animals and is obtained in normal animals only on rare occasions when large doses are given. (3) To elicit acute shock with death even in sensitized animals requires at least a minimal fatal dose of the antigen and usually considerably more is required (two to three times) than the eventual killing dose of the antigen used. (4) Normal guinea pigs that are injected with a moderate dose of anti-

serum, followed within an hour by an injection of antigen, do not differ markedly from normal animals receiving the same amounts of antigen, in regard to acute symptoms. They have occasionally seemed sicker than the normal ones, but not with sufficient regularity to throw any light on any intravascular anaphylactic mechanism.

*Analysis of Bacterial Anaphylaxis in Guinea Pigs.*

We are confronted, therefore, with a problem which cannot be solved entirely by experiments on the living animal. On the one hand, our inability to obtain marked sensitiveness to relatively small doses, unless we allowed a considerable period to elapse between the administration of antiserum and the injection of antigen, points, as in serum anaphylaxis, to the importance of a cellular mechanism. On the other hand, the fact that with larger doses the differences between the sensitized and the normal become less well defined, though still apparent, would indicate that a preparation of the cellular elements by antibody absorption is not absolutely essential.

The alternatives are that either we are dealing with two reactions, one a purely intravascular one which is sufficiently powerful to cause acute shock with death only when very large amounts of antigen are used, and a cellular one which is in evidence only in sensitized animals, or that both in normal and in sensitized animals the mechanism is purely cellular and that the normal tissues of the guinea pig possess originally a slight degree of sensitiveness, analogous to normal antibodies in the circulation, which is merely increased by specific active or passive sensitization.

This problem can be further approached only by recourse to some of the physiological methods, such as heart and lung perfusion lately again applied to serum anaphylaxis by Manwaring, or the study of the isolated uterus—a method which has brought brilliant results in serum anaphylaxis in the hands of Dale<sup>19</sup> and Weil<sup>20</sup> and has lately been applied to the study of bacterial antibodies by Weil and

<sup>19</sup> Dale, H. H., *J. Pharm. and Exp. Therap.*, 1912-13, iv, 517.

<sup>20</sup> Weil, R., *J. Med. Research*, 1912-13, xxvii, 497; 1914, xxx, 317, 331; 1915, xxxii, 107.

Torrey<sup>21</sup> and by Manwaring and Kusama.<sup>22</sup> We accordingly have worked with all these methods but wish to report in this paper only on results obtained with the Dale method.

The first step in this work was to obtain typhoid anaphylactic reactions with the isolated uteri of animals intraperitoneally injected with antityphoid serum. Here again, we found that the interval after injection was much longer than we expected and uteri from these guinea pigs would often react only after 3, 4, and 5 days, several of those tested after 1, 2, and 3 days failing to react. The highest degree of sensitiveness is not acquired after injections of 2 cc. of serum (titer from 1:4,000 to 1:10,000) until about the 5th to the 8th day after injections, a time at which the agglutinins in the circulation of the animal are beginning to diminish. This point of the relation between the circulating agglutinins and the sensitiveness of the whole animal is one of great importance which we believe marks an essential difference between the study of the isolated uterus and the whole animal.

Fig. 1 represents the reaction of a passively sensitized uterus to typhoid antigen when the typhoid bacilli were ground in salt and were extracted with very slightly alkaline salt solution. The bath capacity in these earlier experiments was 200 cc.; in later ones this was reduced to 50 cc. for purposes of economy.

Fig. 2 represents a similar reaction of a highly sensitized uterus where it is shown that the sudden instillation of large amounts of typhoid antigen may give rise to a repetition of spasm, three or four separate shocks being noted. This has not often been the case in our experiments, the uterus usually going into a prolonged single spasm, but was interesting to us because it was a graphic representation of a repetition of shock which we once noticed in young goats on the reinjection of typhoid bacilli. It is also worth noticing that occasionally when a uterus has begun to relax, if more antigen is added, another shock can often be induced even though the first instillation seems to have contained much more antigen than could possibly be absorbed within the uterine cells, a fact which points to the importance of concentration rather than actual amount,

<sup>21</sup> Weil, R., and Torrey, J. C., *J. Exp. Med.*, 1916, xxiii, 1.

<sup>22</sup> Manwaring, W. H., and Kusama, Y., *J. Immunol.*, 1917, ii, 157.

since it is analogous to observations made by pharmacologists in similar experiments with drugs in which the essential element of action seems to depend upon concentration and not upon actual quantity. Repeated addition of the antigen, however, leads to rapidly diminishing intensity of shock and desensitization. Fig. 2 also incidentally seems to show that even when the surrounding antigen is removed, the stimuli which lead to spasm may, on occasion, continue, pointing to the essentially intracellular occurrence of the reaction between antigen and antibody which is the ultimate cause. We should emphasize, however, that the above is the only instance in which such repeated and strong reactions were obtained in this way.

Having thus ascertained that passive sensitization of the animal is easily determined by the Dale reaction, we thought that it would be easy by the same method to determine whether the shock elicited in normal animals by large doses of antigen depended merely upon a lesser degree of cellular sensitization. Were this the case, large amounts of antigen added to a bath containing a normal uterus should induce at least some degree of reaction. Figs. 3 and 4 represent these experiments.

It is apparent from both these charts that large amounts of antigen exert no effect upon the isolated normal uterus. From this it would seem clear that, whatever is the mechanism of injury which induces response of the whole animal to injections of typhoid antigen, it is not one in which cellular sensitiveness is involved, at least, in an anaphylactic sense. This observation we consider of great importance, for it indicates that to the mechanism of bacterial injury in the normal animal, there is superadded another and cellular one in the sensitized animal. It might, of course, be considered that cells other than uterine might be sensitive to direct reaction with the bacterial products in the normal guinea pig. But Weil, especially, has shown a remarkable parallelism between hypersusceptibility in general and uterine reactions in guinea pigs, and in the light of his work and that of others it would seem most likely that the normal animal lacks cellular sensitiveness to the unchanged bacterial substance and that the mechanism of injury, therefore, must be regarded as in large part an intravascular one until the animal is sensitized.

Incidentally, we have shown that with bacterial anaphylaxis the same phenomenon is present that was found in Dale's experiments with horse serum anaphylaxis; namely, that the normal uterus cannot be sensitized, even by prolonged soaking in immune serum, and the union with the antibodies in serum is one in which the antibodies become an intrinsic part of the cells and are not merely physically fixed to the cell exterior.

Up to this point we have worked with bacterial extracts and have found that, in fundamental principles, reactions obtained with bacterial antigen are essentially identical with those obtained with such antigens as horse serum, etc. Inasmuch as the bacteria in infectious diseases are present in the body primarily as living and growing cells, it would seem important to ascertain whether sensitive cells can react to the whole bacterial body, or whether, as we assumed above, there must be a preliminary extraction or solution of the bacteria before injury can be done to the tissue elements. In consequence, we carried out a number of experiments in which heavy suspensions of living bacilli were added to Locke's solution in which was suspended a uterus from a sensitized animal. The opposite horn was, of course, always tested for sensitiveness with typhoid extract. Figs. 5 and 6 illustrate these experiments and show that the whole bacilli exert little or no effect on the sensitized organ.

We must assume, therefore, that where bacteria are injected into the sensitized animal, or are invading the living animal or human body, injury to sensitized tissues must be preceded by a liberation of antigens. Does this occur in the blood stream by lysis? One way of determining this would be to add the serum of a guinea pig injected with whole typhoid bacilli some time before, into a bath containing a sensitized uterus. We tried this, but obtained confusing results owing to the fact that normal guinea pig serum in itself exerts irritating effects upon the isolated guinea pig uterus. In consequence, we resorted to precipitin and complement fixation reactions. The former were not satisfactory, but the latter gave us, we believe, a clear indication of the presence of dissolved antigen in the circulation of guinea pigs intravenously injected an hour previously with whole typhoid bacilli. Such an experiment follows.

*Experiment 7.*—Guinea pig, weight 300 gm., received five slants of living whole bacilli, washed once in salt solution, intravenously. After 1 hour the animal was bled and cultures were taken on agar plates from its whole blood and from the serum after clotting of the blood. The following experiment was then done with the inactivated serum of this animal as antigen; a strong antiserum as antibody; and a fresh guinea pig complement previously titrated carefully against the hemolytic system.

Tube.	Two units of complement added to the following.	Inhibition of hemolysis.
1	0.1 cc. of typhoid antiserum +1.5 cc. of salt solution.....	0
2	0.3 " " serum of injected guinea pig +1.2 cc. of salt solution.....	0
3	0.1 " " typhoid antiserum +0.1 cc. of serum of injected guinea pig +1.4 cc. of salt solution.....	+++
4	0.1 cc. of typhoid antiserum +0.2 cc. of serum of injected guinea pig +1.3 cc. of salt solution.....	++++
5	0.1 cc. of typhoid antiserum +0.3 cc. of serum of injected guinea pig +1.4 cc. of salt solution.....	++++
6	1.6 cc. of salt solution.....	0

To these tubes we added two units of complement and, after 1 hour in the water bath, sensitized red cells were added. The results obtained are expressed in terms of Wassermann reactions as read in 1 hour in the water bath and over night in the ice chest, this being necessary because 0.3 cc. of the injected guinea pig serum was slightly anticomplementary and absolutely complete reactions could not be read until the next morning in this tube and in Tube 5.

There is one possible error which, however, we think we can discount; namely, the culture taken from the whole guinea pig blood in this and other experiments always yielded considerable numbers of colonies of typhoid bacilli, as many as 1,000 or so, which inclines one to consider the possibility that the complement fixation might have been due to whole bacilli. However, the serum from such blood was entirely clear and was further centrifugalized before use, and cultures taken from the serum in this experiment before it was heated for inactivation yielded only two to three colonies per 0.2 cc., which indicated to us that, as one would expect, the great bulk of typhoid bacilli were caught and held in the clot. Had the clot been allowed to stand in the ice chest over night for the expression of the sera, it might still have been possible that an extraction of these typhoid bacilli might have taken place, but we centrifugalized the blood as

soon as clotted and extraction therefore seems unlikely. We believe, therefore, that these experiments indicate that in the circulation of the guinea pig injected with live typhoid bacilli, and presumably, therefore, in the spontaneously infected body, antigen is in some way liberated to the circulating blood, which then could react with sensitized tissue as in our uterus experiment.

If this is the case, the next link in our chain would be to determine whether we could also trace the actual formation of toxic products, such as Friedberger's anaphylatoxin, or Vaughan's split products, or the substances we have spoken of as proteotoxins in the circulating blood. We have attempted to do this in two ways. One consisted in repeated attempts to produce acute symptoms of shock in guinea pigs by injecting intravenously blood serum taken from normal animals rendered severely sick both by typhoid bacilli and typhoid extract. We have never succeeded in this. Next we attempted to produce contraction in the normal guinea pig uterus, by adding to the bath of Locke's solution such guinea pig serum. In this we did not succeed, partly because it was never possible to add large amounts of guinea pig serum to these baths without running the risk of obtaining the contraction incident to the instillation of normal guinea pig serum. When we did so, no marked contrast appeared between the result of putting in normal guinea pig serum and putting in the same amount of the serum of an animal dying of typhoid bacilli (Fig. 7).

We cannot, therefore, with any of the methods now at our disposal, demonstrate the formation in the blood stream of the typhoid-infected animal of the poisons which are responsible for acute anaphylactic shock. Does this negative the intravascular production of such toxic substances? We think not.

In the first place, it must be remembered that, whatever may be the mechanism of injury going on in an animal infected and poisoned with typhoid or other bacilli, the process is a gradual one extending over a period of from 4 to 12 hours or longer, even when large doses are given. It is likely that the poisons which are formed are absorbed with considerable speed, this being responsible for the symptoms developing in the animal. It is not likely, therefore, that we could find at any one time a sufficient amount of the toxic substance present in the limited quantity of blood serum which we can readily inject into

an animal or instil into the Dale's bath, to expect to obtain acute reactions. For with bacterial anaphylaxis especially, it must be remembered that acute shock is obtained with no great regularity and only in animals highly sensitized when considerable quantities of bacterial antigen are administered.

Figs. 8 and 9 demonstrate incidentally that the conditions observed with bacterial antigens are to a great extent similar to those observed when other cellular antigens, such as erythrocytes, are used. Fig. 8 shows a typical reaction with the uterus of a guinea pig which had received 2 cc. of amboceptor intraperitoneally 3 days before, when hemolyzed red cells were added to the bath, and also indicates subsequent desensitization; and Fig. 9 shows the complete failure of reaction when the cells are added as "whole" cells unhemolyzed to a similarly sensitized organ. Reaction can be elicited by cells hemolyzed with distilled water or hemolyzed directly in the Locke's bath by the addition of amboceptor and complement, thus showing the preliminary function of solution of the cells in the blood stream carried out by the circulating antibodies. We do not insert a chart to show this because these records are very long owing to the necessity of allowing plenty of time between the instillation of amboceptor, complement, and red cells, to eliminate non-specific irritation of the uterus by these substances separately. The results were clear-cut, contraction usually beginning at about the time when hemolysis began and progressively increasing towards its completion.

Another point which formed an important part of our scheme of work was the study of the possible influence of circulating antibodies upon the reaction between antigen and sensitized tissues. To obtain light on this subject, we proceeded by the following two methods. In one series of experiments antiserum was injected into sensitized animals just before the injection of antigen, in order to ascertain whether by this means shock was diminished or eliminated. In another series, antigen was incubated for from 1 to 3 hours with antiserum and the mixture instilled into Locke's baths containing sensitized uteri.

An experiment by the former manner of procedure is as follows:



*Experiment 8.*—Apr. 7, 1917. 2.30 p.m. Passively sensitized guinea pig, weight 190 gm., receives intravenously 0.5 cc. of strong typhoid antiserum. After 14 minutes receives 3 cc. of typhoid antigen. Very slight symptoms; breathing slightly labored but runs about immediately and shows hardly any illness for the first 10 or 15 minutes.

Passively sensitized guinea pig of the same lot as the preceding animal. Agglutinin titer about 1:160. Receives 3 cc. of typhoid antigen. Very sick immediately; coughs and breathes irregularly. Much sicker than the preceding guinea pig.

These two animals represent a few of a considerable number similarly treated. With a few exceptions it seemed fairly definite that the presence of a considerable concentration of antibodies in the circulation delayed rather than hastened the development of acute symptoms.

The explanation for this we think is to be found more definitely in the following experiments with isolated uteri.

Fig. 10 represents an experiment in which the typhoid antigen which in substance consisted of a turbid extract containing not only extracted dissolved substances, but also particles, was exposed to the action of antiserum until a heavy precipitate had formed. This precipitate was then washed and added to the Locke's bath. No reaction resulted. The clear supernatant fluid, however, gave a sharp reaction in all cases, whether or not we added an excess of antiserum and reincubated.

Inasmuch as our antiserum gave only a slight precipitation with clear, filtered typhoid extract, it remained uncertain whether or not the experiment represented by Fig. 10 meant only that undissolved particles had been precipitated or whether it also signified that a part of the dissolved antigen was rendered useless by the antibodies. After several unsuccessful attempts to obtain a clear answer to this query by working with typhoid antigen and antibody, we decided to repeat the experiment using human serum as antigen and antihuman rabbit serum as antibody. Fig. 11 represents such an experiment.

Here 0.5 cc. of human serum in 1 cc. of salt solution was incubated with 0.3 cc. of a strong antihuman rabbit serum. A heavy precipitate formed which was thrown down in the centrifuge and washed once with salt solution, then shaken up in a small amount of salt solution and added to the bath containing a passively sensitized uterus. No

TABLE I.

Date.	Antigen.	Normal guinea pigs.		Sensitized guinea pigs.	
		Weight.	Remarks.	Weight.	Remarks.
1916	cc.	gm.		gm.	
Mar. 31	3	240	Lives.	250	Sensitized Mar. 27. No immediate symptoms; dead next day.
May 5	2	227 205	Dead next a.m. Dead 5 to 6 hrs.	215 215	Immediate. Sensitized Mar. 31. Symptoms marked; dead next a.m.
Apr. 13	2	195	Sick but no shock; died next a.m.	190	Sensitized on Apr. 8. Definite shock; dead same afternoon.
	1	180	No symptoms; lives.	180	Mild shock; dead same afternoon. Agglutinations of guinea pigs of same series up to 1 : 160.
Apr. 14	3 (ureter).	180	Immediate shock-like symptoms; dead next a.m.	180	Sensitized Apr. 8. Typical shock; dead next a.m. Sensitized sick more rapidly; mate agglutinin, 1 : 320.
Apr. 15	3	190	No shock; dead next a.m.	180	No shock; lives. Mate agglutinin, up to 1 : 80.
Oct. 24	1 (strong).	220	Slight symptoms; lives.	220	Sensitized Oct. 28. No shock; dead next a.m. Mate agglutinin, up to 1 : 100.
1917					
Jan. 19	4	*	Immediately sick; died next a.m.	*	Sensitized Jan. 1. Heavier than control. Shock; died in 4 hrs. Mate agglutinin, 1 : 100.
	5	200	Distinct moderate shock; died next a.m.	170	5 cc. Acute shock; died.
Apr. 4	25	300	No symptoms; died next a.m.	270	Severe shock; lives. Agglutinin, 1 : 80.

\*Error in records; no note of weights made.

reaction occurred. However, again the addition of the supernatant fluid to the same bath resulted in a strong reaction.

This experiment was in entire harmony with those performed with typhoid antigen and antibody. It showed that antigen precipitated by antibody was thereby neutralized as far as its powers to react with sensitized tissues were concerned. It also showed, incidentally, a fact immunologists have known for some time, that it is extremely difficult to remove all antigen from solution by precipitation with antibody, and that most of the substance of a specific precipitate is derived from the antiserum.

We still wished to determine if possible, whether an animal sensitized to typhoid protein was or was not thereby rendered more vulnerable; *i.e.*, more easily killed by typhoid antigen. Table I, constructed from scattered observations made from time to time throughout our work gives some clue to the question, though it by no means settles the matter conclusively.

It will be seen that as a rule the sensitized animals died more quickly than did the normal controls, in spite of the fact that they were slightly protected by the presence of small amounts of antibody. This point will need further experimentation, which is already in progress.

#### SUMMARY.

We have attempted in the preceding experiments the beginning of an analysis of bacterial anaphylaxis and its relation to the occurrences in the animal body during an infectious disease. We have shown that the sensitization of the tissues of guinea pigs, as indicated by the isolated uterus, required 3 to 5 days even when passive sensitization was employed, and that in these relations conditions with bacterial sensitization were entirely analogous to those revealed for serum anaphylaxis by Dale and Weil especially. It has become apparent that the sensitized uterus reacted not at all with whole bacteria or whole red cells, or, in other words, that before reaction with sensitized organs could occur an extraction or solution of the bacterial cell must take place. That bacteria yield some of their substance to the circulating blood during the course of infection was to be expected, but it has been definitely indicated, we think, by our complement fixations.

The mechanism of injury in the sensitized animal or in the human being so far along in typhoid fever that antibodies have begun to develop is in part one in which antigen, derived from the bacilli and brought into solution, or rather suspension, in the blood stream, reacts with antibodies which are from the beginning, or have subsequently become, integral parts of the cell protoplasm, the entire process taking place within the cell. This last point is indicated by the failure to sensitize by simply soaking the normal uterus in antiserum.

This, however, cannot be the entire story of injury. We know that typhoid antigen injected into normal animals in moderate amounts will render them gradually sick and eventually kill them. Also, a sufficient amount injected into a normal animal will occasionally produce acute symptoms, in every respect similar to the reaction produced in sensitized animals by smaller doses. We have shown that such acute symptoms in normal animals were not due in any degree to tissue sensitiveness, since even very large quantities of antigen will produce no response on the part of the normal uterus. It is reasonable to suppose, therefore, that the injury, gradual or acute, in the normal animal, is in no respect referable to tissue sensitiveness to the whole antigen, but rather must be referred to some series of phenomena which occur in the circulation. The acute shock of normal animals may possibly, therefore, be entirely due to an intravascular reaction. Whether this is one of antigen-splitting, or of antienzyme removal in the sense of Jobling is a point on which these experiments throw no light.

It is true that we have never succeeded in producing acute toxic symptoms either in the whole animal or in the isolated uterus with serum from animals acutely ill. This we eliminate as negative evidence inasmuch as we believe that the toxic substances need at no given time be present in the blood stream in sufficient concentration to render such an experiment successful. They are probably absorbed and do their injury almost as rapidly as formed, an assumption which is based on the speed with which symptoms develop.

It is possible, and not to be denied on the basis of any experiment that we can devise at present, that the gradual illness of the normal animal and the occasional acute shock of these animals may be based on entirely different mechanisms. In both cases, however,

in normal animals, they seem to be intravascular. And since the symptoms of acute shock which can be produced in sensitized animals with moderate doses can also, though only occasionally, be produced in normal animals with larger doses, it is reasonable to suppose that the poisons produced intracellularly in the one may be similar to those produced intravascularly in the other.

It does not seem likely that the specific circulating antibodies are in any way sources of increased injury to an animal spontaneously infected with bacteria. If sufficiently powerful at the beginning they may even prevent tissue injury, first by increasing phagocytosis, then by producing intravascular agglutination, and finally, as indicated by our experiments, even by removing a part of the antigen from possible reaction with the cell, though in this last respect our experiments indicate that they functionate imperfectly. It is more probable that their chief protective action to the sensitized body lies in removing the whole bacteria from the possibility of intravascular disintegration, which, as we have shown, is prerequisite to anaphylactic injury of the tissues of the host.

We would tentatively summarize our opinion as to the occurrences in the typhoid-infected body as follows: Early injury is probably due to disintegration of part of the bacteria in the course of which albumose-like bodies are liberated, and, following which, intravascular reactions result in the formation of toxic substances, perhaps by some form of proteolysis.

Since the accumulation of bacteria during these stages is relatively slight, this form of injury probably plays little part in producing symptoms. Indeed, the experiment by which acute injury is produced in the normal guinea pig by the sudden injection of several times the lethal dose of partly dissolved bacteria, finds no analogy in the spontaneously diseased body.

At this time the tissues are not sensitive, but as antigen absorption progresses and the tissues are stimulated to react, sensitiveness develops, which renders them much more delicately amenable to injury by direct reaction with even small amounts of dissolved but otherwise unaltered antigen. This process is directly counteracted by circulating antibodies which tend to remove the bacteria from the possibility of yielding their antigen to solution by agglutinating

them, aiding phagocytosis, and to a slight extent even neutralizing dissolved antigen.

It seems likely, therefore, that the symptoms which appear as the incubation time ends are largely those due to cellular sensitization which probably begins before any considerable amount of circulatory antibodies is present. The circulating antibodies would seem to have little or nothing to do with intravascular injury, the ferments responsible for this, however much it may occur, probably consisting of the non-specific proteases studied in this connection by Jobling.

Finally it appears that highly sensitized animals are more easily killed by typhoid antigen than are normal animals, provided they do not dispose over unusually large amounts of circulating antibodies.

Cure would consist of a gradual checking of growth and final destruction of the bacteria, and the consequent cessation of antigen liberation, but delicate hypersusceptibility would probably persist for some time after cure and immunity have been established. Just what the relation between tissue hypersusceptibility and immunity is remains a problem for further study.

#### EXPLANATION OF PLATES.

##### PLATE 38.

FIG. 1. Typical reaction of sensitized uterus to typhoid antigen.

FIG. 2. Repeated spasm on inoculation of large amounts of antigen. A reaction like this was obtained very exceptionally. No other was obtained in which the same degree of repetition was noticed.

FIG. 3. Failure of normal uterus to react to typhoid antigen. This uterus had been soaked in immune serum for  $3\frac{1}{2}$  hours.

FIG. 4. Failure of normal uterus to react to typhoid extract in spite of the repeated inoculation of large amounts. The amounts instilled in the bath in this case were at least four times greater than sufficient to kill a guinea pig of the same size.

FIG. 5. Failure of sensitized uterus to react to large doses of living typhoid bacilli.

##### PLATE 39.

FIG. 6. Another instance of the failure of living typhoid bacilli in large amounts to stimulate sensitized uterus.

FIG. 7. Failure of normal uterus to react when the serum of a guinea pig dying of living typhoid bacilli was added. Attempt to demonstrate proteotoxin in serum of a typhoid-infected animal.



FIG. 1.



FIG. 2.



FIG. 4.



FIG. 3.



FIG. 5.







FIG. 6.



FIG. 7.



FIG. 8.



FIG. 9.



FIG. 10.



FIG. 11.



FIG. 8. Typical reaction of the uterus of a guinea pig passively sensitized with amboceptor when hemolyzed red cells are added.

FIG. 9. Uterus of a guinea pig passively sensitized to red cells. The chart shows the failure of reaction when cells are intact and reaction when cells are disintegrated by hemolysis.

FIG. 10. Uterus passively sensitized to typhoid antigen. The chart shows the failure to react when precipitate produced by the action of antiserum is added. Reaction when supernatant fluid of such a mixture is added.

FIG. 11. Uterus passively sensitized to human serum. Failure of reaction when precipitate produced by reaction of human serum and antihuman serum is added, but prompt reaction when the supernatant fluid of such a precipitin reaction is instilled into the bath.



## INCREASED VIRULENCE OF THE HOG-CHOLERA BACILLUS PRODUCED BY PASSAGE THROUGH RABBITS.

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In a previous paper (1) the writer mentioned the attempt to modify the carbohydrate reactions of a culture of the hog-cholera bacillus by passing it through a series of rabbits. The only change noted was an increase in virulence, and it is the object of this paper to record this change.

Since the time of Pasteur many organisms have had their virulence increased by animal passages but the recorded results of the passage of the hog-cholera bacillus through rabbits are somewhat conflicting. Moore (2) passed a typical organism through a series of twenty-six rabbits and concluded that there was no increase in virulence for this animal. He judged the virulence by the time taken to kill the animal and did not consider the amount of culture necessary to produce death. Previous to this Selander (3) had reported the rapid increase in virulence of a hog-cholera bacillus by rabbit passages and his work was apparently confirmed by Metchnikoff (4). Smith and Moore (5) had shown, however, that Metchnikoff was working with the swine-plague bacillus and as Moore could not confirm the work of Selander he concluded that the latter was probably also working with the swine-plague organism.

Smith (6) later worked with a culture of the hog-cholera bacillus of low initial virulence further attenuated by age, which would not kill rabbits when injected under the skin. By animal passages, he increased the virulence to the point where subcutaneous injections were fatal.

Later Smith and Reagh (7) in attempting to modify the agglutinability of a strain of the hog-cholera bacillus, passed it through a series of fourteen rabbits. As a result of this passage the virulence was increased from a minimal fatal dose of 0.1 cc. in the stock strain to 0.00001 cc. in the passage strain. It has already been pointed out (1) that this increased virulence still persists to a certain degree after a lapse of 15 years.

The organism with which we worked is a stock culture known as Hog-cholera XII. It was isolated by Dr. Smith in 1914 from the spleen of a pig dying from hog-cholera. Soon after this it was passed through a rabbit and since that time it has been kept on slant agar in the cold, transfers being made monthly.

Culturally it is a motile, Gram-negative rod, growing readily on the ordinary media and forming acid and gas in dextrose bouillon but not attacking lactose or saccharose. It is quantitatively agglutinated by serum from rabbits injected with other strains of the hog-cholera bacillus and when injected into animals it causes the production of agglutinins for other strains of the hog-cholera bacillus.

Starting in January 1916 this culture was passed through a series of eleven rabbits; the essential details of this passage will be found in Table I. The passage was made directly from one animal to the next by using a suspension of crushed spleen for the inoculation. After the third transfer the inoculation was made by rubbing a small amount of the spleen suspension into the shaven skin of the next rabbit.

TABLE I.  
*Passage of Hog-Cholera XII through Rabbits.*

Pas- sage.	Weight of rab- bit.	Method of infection.	Material and dose.	Length of life.	Remarks.
	<i>gm.</i>			<i>days</i>	
1	1,199	Subcutaneous.	0.5 cc. of 24 hr. bouillon culture.	6	Typical lesions.
2	1,172	"	Spleen suspension.	6	" "
3	1,274	"	" "	7	" "
4	1,305	Cutaneous.	" "	7	" "
5	1,430	"	" "	7	" "
6	2,073	"	" "	9	" "
7	2,089	"	" "	10	" "
8	2,273	"	" "	8	" "
9	2,029	"	" "	7	" "
10	2,308	Subcutaneous.	0.000001 cc. of 24 hr. bouillon culture from Rabbit 9.	9	" "
11	2,336	"	0.00000001 cc. of 24 hr. bouillon culture from Rabbit 10.	6	" "

It would be difficult to estimate accurately the relative virulence of this culture from the duration of the disease in the various animals but a comparison of the number of organisms necessary to kill gives us very definite results. With the stock culture one must use somewhat over 0.00001 cc. of a 24 hour bouillon culture to produce death.

Of four rabbits given a subcutaneous injection of this amount one died in 12 days, one showed an increased temperature and loss in weight, the other two showed no effects. One rabbit that was given 0.001 cc. subcutaneously died in 4 days. With the strain of the same bacillus passed through the rabbits, 0.00000001 cc. of a 24 hour bouillon culture injected subcutaneously into a rabbit weighing 2 kilos causes death in about 6 days. The passage strain has therefore been increased in virulence about one thousand times and plate counts show that the number of organisms necessary to infect has been reduced from approximately 20,000 to 20.

The type of disease produced by the more virulent organism shows no striking departure from that caused by the original culture. This organism seems to have a greater power of penetration than do most cultures of the hog-cholera bacillus, as it causes only a slight local lesion. The bacteria apparently enter the body through the lymphatics, for the axillary and inguinal lymph nodes on the side of the inoculation are enlarged and congested and often show large areas of necrosis.

The passage through rabbits has produced no change in the morphology of the organism that can be detected either in films made from the spleen of an animal dying from infection or in films made from cultures. The passage strain is slightly more motile than the original culture but this difference is not marked. It is also more susceptible to agglutinins, in that clumps are formed in a shorter time, but the readings after 2 hours' incubation are the same. The passage of the original strain through one rabbit makes it as susceptible to agglutinins as is the strain passed through eleven rabbits. With some immune sera the parent strain is agglutinated in slightly higher dilutions than is the passage strain but here too the difference is not great.

#### SUMMARY AND CONCLUSION.

By passage through a series of eleven rabbits a culture of the hog-cholera bacillus has increased its virulence a thousand times. A subcutaneous injection of twenty organisms, or 0.00000001 cc. of a 24 hour bouillon culture, or a drop of a bouillon culture rubbed lightly into the shaven skin, produces, in the rabbit, a characteristic disease resulting in death on or about the 6th day.

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# THE SIGNIFICANCE OF AGGLUTININS IN THE IMMUNITY OF THE RABBIT TO THE HOG-CHOLERA BACILLUS.

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A great deal of experimental work has been done on agglutinins, yet we have very little evidence as to the part they play in immunity to any given infectious disease. Much of the work tends to show that they are not indicators of immunity but in spite of this the agglutinin titer is often used as an index of immunity after antityphoid vaccination and when the titer falls it is regarded as an indication for revaccination. In the production of various immune sera it is often assumed that the height of the agglutination titer is an index of the antibody content but the evidence on which this assumption is based is not clear. We have endeavored to throw some light on the relation of agglutinins to immunity by the use of the highly virulent culture of the hog-cholera bacillus described in the previous paper (1).

A large part of the experimental work on agglutinins has been done with the rabbit and the horse inoculated with the typhoid bacillus, and the great weakness of this work is that in the former animal at least, this organism does not produce a true invasive disease but acts only when large numbers of bacteria are given. On the other hand, the hog-cholera bacillus produces, in the rabbit, a true disease resembling in many respects typhoid fever in man.

## HISTORICAL.

In 1894 Smith and Moore (2) showed that heated cultures of the hog-cholera bacillus would not produce an immunity in the rabbit but that the injection of a living attenuated culture would produce an active immunity to the more virulent organism. They did not at this time study the agglutinins, but later Smith and Reagh (3) produced agglutinins by the injection of heated as well as living cultures of the hog-cholera bacillus.

Shoukévitch (4) reports work similar to that to follow in which, using the hog-cholera bacillus and the rabbit, he showed that the injection of heated cultures caused an increase in the complement-fixing bodies, agglutinins, and opsonins but that this was not associated with an immunity. The injection of a living culture of low virulence caused a very slight production of these bodies but did cause an active immunity to more virulent organisms. Of fifteen animals tested in this way, five showed no effects, one died with a typical septicemia, two died from acute intoxication, and seven died in from 15 to 70 days with a paralysis of various groups of muscles. All showed little or no increase in the immune bodies following the injection of the culture of low virulence.

Whether the phenomenon described by Bull (5) under the name of *intra vitam* agglutination is related to *in vitro* agglutination is a question that will not be considered in this paper but we will consider the relation of this phenomenon to immunity to the hog-cholera bacillus. Bull has recorded experiments with a rather large series of organisms, the avirulent members of which are clumped when injected intravenously into normal rabbits, while the virulent members are not clumped and are found in the circulation for some time after the injection. When the bacilli are clumped they rapidly disappear from the blood stream and in a half hour after the injection, films from the liver and lungs show many polymorphonuclear leukocytes filled with organisms. Virulent organisms are not clumped in the circulation of normal rabbits but are rapidly clumped and phagocyted when injected into immune animals. Bull says (5) "The degree of agglutination and opsonization of bacteria within the animal body is inversely parallel to the infectiousness of the bacteria for the host" but he is careful to state that exceptions may be found to this rule.

#### EXPERIMENTAL.

*Cultures Used.*—Reference has already been made to the highly virulent test culture (1). The parent culture that had not been passed through rabbits and having the laboratory number XII was used as a vaccine and in small doses of the living culture to produce an immunity.

Hog-cholera Neb., isolated by Dr. Smith in 1886 (6), is now a culture of very low virulence. It is a typical hog-cholera bacillus except for its lessened virulence.

Hog-cholera Ark., isolated by Dr. Dinwiddie in 1889, was referred to by Dr. Smith in 1903 (3) and the cultural characters have been reported more recently (7). It is an organism of moderate virulence, probably due in part at least to its prolonged cultivation on artificial media.

*Use of Heated Cultures.*

The first experiment was made with a vaccine prepared by suspending the 24 hour growth from an agar slant inoculated with Hog-cholera XII in 10 cc. of salt solution and heating for 1 hour at 60°C. The heated suspension was incubated over night and several loops were transferred to bouillon in order to be sure that it was sterile. Having proven that all the bacteria had been killed the vaccine was injected subcutaneously into two rabbits of approximately equal weight. The first injection was of 0.5 cc. and was followed in 5 days by 1 cc. 6 days later they were each given 2 cc. Both rabbits bore the injections well, showing only a slight and temporary loss in weight.

18 days after the beginning of the treatment and 7 days after the last inoculation these two rabbits, together with a control, were bled from the ear vein and the serum of the inoculated animals was tested for agglutinins against the strain of the hog-cholera bacillus with which they had been injected and also against the strain that had been passed serially through rabbits and with which they were to be inoculated in order to test their immunity.

In order that the rabbits might have every opportunity to show any immunity they might have gained by the injection of the heated cultures they were tested by the cutaneous method, two drops of a 24 hour bouillon culture being lightly rubbed into the shaven skin of each animal. This test was made 2 days after the bleeding from the ear vein and 9 days after the last injection of the heated cultures. The results of this experiment are summarized in Table I.

TABLE I.

*Test of the Power of Heated Cultures to Produce Agglutinins and Immunity.*

Rabbit No.	Vaccinated.	Limit of agglutination* for Hog-cholera XII.		Weight.	Immunity test. Result of cutaneous inoculation with test culture.
		Stock.	Passage strain.		
1	Three times with heated culture.	$\frac{1}{12,800}$	$\frac{1}{12,800}$	1,823	Died in 5 days.
2	Same as No. 1.	$\frac{1}{12,800}$	$\frac{1}{12,800}$	1,889	" " 7 "
3	Control.	Not tested.		2,006	" " 6 "

\* The limit of agglutination is the highest dilution in which clumps of bacteria can be seen with the naked eye. A 24 hour bouillon culture was used as an antigen throughout.

It will be seen that while both treated rabbits had in their serum agglutinins that could be demonstrated in a dilution of  $\frac{1}{12,800}$  they

had no immunity. Both died in approximately the same number of days after the inoculation as did the control. At autopsy all three rabbits showed the same local as well as visceral lesions, the only essential difference being that both vaccinated rabbits had a small area of pneumonia. It is quite possible, however, that the latter was due to another organism that was affecting our stock rabbits.

A similar experiment, but with results that are not so clear-cut, was made some time later. The preparation of the vaccine and the dosage was the same as in the preceding experiment except that the culture used was the highly virulent rabbit passage strain. The test for immunity was made by injecting subcutaneously 0.0000001 cc. of a 24 hour bouillon culture of the passage strain. The results are summarized in Table II and show that of the vaccinated rabbits the one that had the higher agglutination titer died, while the other one survived without showing any marked effects from the inoculation. Examination of the controls shows that we were using about the smallest dose that would cause an infection, so that the apparent immunity of the one vaccinated rabbit might be due to causes other than the injected vaccine such as a natural immunity or too small an infecting dose. In spite of this discordant result it is clear that there was no demonstrable immunity in the rabbit with the higher agglutination titer.

TABLE II.

*Test of the Power of Heated Cultures to Produce Agglutinins and Immunity.*

Rabbit No.	Vaccinated.	Limit of agglutination for test culture.	Weight.	Subcutaneous injection of test culture.	
				Amount.	Result.
			gm.	cc.	
4	Three times with heated culture.	$\frac{1}{25,600}$	2,011	0.0000001	Died in 9 days.
5	Same as No. 4.	$\frac{1}{12,800}$	2,030	0.0000001	No effect.
6	Control.	0	2,683	0.0000001	Died in 9 days.
7	"	0	1,763	0.00000001	No effect.

*Use of Living Cultures to Produce Immunity.*

The immunization of rabbits by the injection of living cultures of the hog-cholera bacillus is very difficult, for their resistance may be overcome by the injection of too large doses or they may succumb to

some spontaneous disease to which they seem very susceptible during such treatment. We did succeed in getting four animals to the stage where they could be tested against the highly virulent culture and as the treatment of each animal is different from the others a summary of the immunization is given below.

*Rabbit 8.*—Three injections of a non-virulent strain followed by a mildly virulent strain.

June 6, 1916. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Slight rise in temperature but no loss in weight.

June 20. Subcutaneous injection of 1 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Loss in weight but no increase in temperature.

July 8. Intravenous injection of 0.1 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Rise in temperature but no loss in weight. Another rabbit that had had the same injections died during the night following the intravenous inoculation.

July 21. Subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture of Hog-cholera Ark. No loss in weight and no rise in temperature. Previous tests had shown that this culture in this amount would kill a normal animal in 20 days.

This rabbit was bled three times after the last inoculation and the results of the agglutination tests are given in Table III.

TABLE III.  
*Agglutination Titer of the Serum of Rabbit 8.*

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 4	Sept. 19	Oct. 3
Hog-cholera XII .....	$\frac{1}{80,000}$	$\frac{1}{51,200}$	$\frac{1}{3,200}$
“ XII, passage series .....	$\frac{1}{20,000}$	$\frac{1}{25,600}$	$\frac{1}{6,400}$

*Rabbit 9.*—Injected with increasing numbers of Hog-cholera Ark. (mildly virulent strain).

June 6, 1916. Subcutaneous injection of 0.0001 cc. of a 24 hour bouillon culture. Rise in temperature but no loss in weight.

June 20. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture. Rise in temperature and loss in weight extending over a period of 20 days.

July 21. Subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture. Slight loss in weight but no rise in temperature.

This last injection was one that would kill the normal rabbit, so no further injections were given but the animal was allowed to rest until it was tested for immunity to the highly virulent strain. During this period of rest it was bled three times and the limit of agglutination for the hog-cholera bacillus determined. The results of these tests are given in Table IV.

TABLE IV.

*Agglutination Titer of the Serum of Rabbit 9.*

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 4	Sept. 19	Oct. 3
Hog-cholera XII .....	$\frac{1}{20,000}$	$\frac{1}{3,200}$	$\frac{1}{1,600}$
“ XII, passage series .....	$\frac{1}{5,000}$	$\frac{1}{1,600}$	$\frac{1}{1,600}$

*Rabbit 10.*—Injected with increasing amounts of Hog-cholera XII (virulent culture).

June 20, 1916. Subcutaneous injection of 0.0000001 cc. of a 24 hour bouillon culture. Rise in temperature but no loss in weight.

July 11. Injection of June 20 repeated. No rise in temperature and no loss of weight.

July 21. Subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture. No loss in weight or rise in temperature.

Aug. 7. Subcutaneous injection of 0.001 cc. of a 24 hour bouillon culture. No effect on weight or temperature. Control rabbit died in 4 days.

Aug. 19. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture. No effect on weight or temperature. The animal was now allowed to rest for some time before its resistance to the highly virulent strain was tested. Table V gives the results of the agglutination tests made during this period.

TABLE V.

*Agglutination Titer of the Serum of Rabbit 10.*

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 31	Sept. 17	Oct. 3
Hog-cholera XII .....	$\frac{1}{25,600}$	$\frac{1}{25,600}$	$\frac{1}{6,400}$
“ XII, passage series .....	$\frac{1}{25,600}$	$\frac{1}{25,600}$	$\frac{1}{6,400}$

*Rabbit 11.*—One injection of a sublethal number of Hog-cholera XII bacilli (virulent culture).

Aug. 22, 1916. Subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture of Hog-cholera XII. Marked loss in weight and rise in temperature. Another rabbit inoculated with the same amount of culture at the same time died in 12 days. In Table VI are given the results of the agglutination tests made on the serum of this rabbit previous to its inoculation with the highly virulent culture.

TABLE VI.

*Agglutination Titer of the Serum of Rabbit 11.*

Culture agglutinated.	Limit of agglutination for serum drawn on.	
	Sept. 18	Oct. 3
Hog-cholera XII .....	$\frac{1}{6,400}$	$\frac{1}{3,200}$
“ XII, passage series .....	$\frac{1}{3,200}$	$\frac{1}{3,200}$

On Oct. 3 these four rabbits together with two controls were bled from the ear vein and the sera tested for agglutinins to the highly virulent culture. 3 days later each was given a subcutaneous injection of 0.000001 cc. of a 24 hour bouillon culture of the highly virulent organism in order to test their immunity. The necessary data for the understanding of this test together with the results are given in Table VII. The subcutaneous route was chosen for the inoculation in order to be sure that all the animals received the same amount of culture.

Examination of the table will show that the rabbits previously injected with living cultures were not affected when inoculated with the highly virulent culture, whereas the controls died in 7 days. The agglutination titer of the sera of each of these immune animals was below that found in the serum of the animals injected with heated cultures, yet the latter promptly succumbed to an inoculation with this virulent culture in small amounts.

3 months later two of these immune rabbits were again tested for agglutinins and then injected with a large amount of the highly virulent culture. The results are summarized in Table VIII where it will be seen that one rabbit did not survive this severe test while the animal with the lower titer resisted one thousand times the minimal fatal dose.

TABLE VII.

*Test of the Power of Living Cultures to Produce Agglutinins and Immunity.*

Rabbit No.	Immunization.				Test of immunity to Hog-cholera XII, passage series, on Oct. 6, 1916.			
	Date of injection.	Strain of hog-cholera.	Amount of 24 hr. bouillon culture injected.	Route.	Time after last injection.	Weight.	Agglutination titer 3 days before test of immunity.	Result of subcutaneous injection of 0.000001 cc. of 24 hr. bouillon culture.
	1916		cc.		days	gm.		
8	June 6	Neb.	0.01	Subcutaneous.	77	3,028	$\frac{1}{6,400}$	No effect.
	" 20	"	1.0	"				
	July 8	"	0.1	Intravenous.				
	" 21	Ark.	0.1	Subcutaneous.				
9	June 6	Ark.	0.0001	Subcutaneous.	77	3,374	$\frac{1}{1,600}$	No effect.
	" 20	"	0.01	"				
	July 21	"	0.1	"				
10	June 20	XII	0.0000001	Subcutaneous.	48	2,768	$\frac{1}{6,400}$	No effect.
	July 11	XII	0.0000001	"				
	" 21	XII	0.00001	"				
	Aug. 7	XII	0.001	"				
	" 19	XII	0.01	"				
11	Aug. 22	XII	0.00001	Subcutaneous.	45	2,024	$\frac{1}{3,200}$	No effect.
12	Normal animal for control.				0	2,932	0	Died in 7 days. Typical lesions.
13	Normal animal for control; injection one-tenth the amount given other rabbits.				0	2,359	0	Died in 7 days. Typical lesions

The results of these tests clearly indicate that an animal may show agglutinins to the hog-cholera bacillus *in vitro* and yet have no immunity. It cannot be said, however, that these bodies have no relation to immunity for they are present in the sera of all the immune animals though, at the time of the test, not in as high dilutions as in the vaccinated rabbits.



TABLE VIII.

*Test of the Power of Living Cultures to Produce Agglutinins and Immunity.*

Rabbit No.	Immunization.	Agglutination titer 3 days before test of immunity.	Test of immunity.	
			Amount of 24 hr. bouillon culture injected.	Result.
8	See Table VII.	$\frac{1}{1,280}$	0.0001	Died in 8 days.
11	" " VII.	$\frac{1}{640}$	0.0001	Slight rise in temperature.
6	Control.	0	0.0000001	Died in 9 days.
7	"	0	0.00000001	No effects.

Attempts have been made to differentiate after the method of Joos (8) the agglutinins in the vaccinated rabbit from those found in the immune animals. No difference has been found in the susceptibility to heat of the agglutinins from these two sets of animals nor do the sera act differently on heated bacteria.

#### *Intra Vitam Agglutination.*

The work of Bull (5) suggested the comparison of the *intra vitam* agglutination in vaccinated rabbits and in those immunized by the use of living cultures. When a suspension of the living organisms from the highly virulent strain are injected intravenously into either of these animals they are promptly clumped and rapidly disappear from the circulation. Films made from the liver half an hour after the injection show cells packed with bacteria. Most of the phagocytic cells found were polymorphonuclear leukocytes but a few phagocytic endothelial cells were also present. No difference was noted in the reactions of animals from the two sets though one was immune and the other was very susceptible to the bacterium injected. The most interesting phase of this work was that the control animals also showed typical *intra vitam* clumping. This fact was verified repeatedly but only one experiment will be given.

The growth from two 24 hour agar slants inoculated with Hog-cholera XII passage virus was suspended in 15 cc. of salt solution. Shaken and centrifugal-

ized for  $\frac{1}{2}$  hour. Removed the supernatant fluid and suspended the residue in 5 cc. of salt solution and shook vigorously to break up clumps. Injected intravenously into a normal rabbit weighing 2,270 gm. Blood removed from the heart at stated intervals and dilutions made for plate cultures. The first dilution was made in a glass-stoppered bottle and was shaken for some time to break up clumps. At the same time films were made that later were stained with Manson's methylene blue. The findings in the films as well as the results of the plate counts are given in Table IX.

40 minutes after the injection, the rabbit was chloroformed and films were made from the liver. Cells containing bacteria were present but were not numerous.

TABLE IX.

*Intra Vitam Agglutination Test Using a Normal Rabbit.*

Time after injection.		Bacteria per cc. in heart's blood.	Result of examination of film.
min.	sec.		
0	31	30,000,000	Large clumps of bacteria embedded in a blue-staining homogeneous mass.
2	30	4,800,000	No bacteria found.
5	24	5,950,000	" " "
15	0	75,000	" " "
30	0	117,000	" " "

This experiment, as well as the others made, shows that in the normal rabbit there is a prompt clumping of the injected bacteria, a rapid disappearance from the blood stream as shown by films and plate counts, and a phagocytosis of the bacteria by cells in the liver and in other organs. The centrifugalization of the bacteria had nothing to do with the clumping for it occurred in other animals where a suspension made directly from the agar slant was injected. When the dilutions of blood were not shaken in glass-stoppered bottles, plates made 30 minutes after the injection and containing  $\frac{1}{500}$  cc. of blood were sterile.

#### SUMMARY AND CONCLUSION.

Rabbits may show a high agglutination titer to the hog-cholera bacillus and have no immunity and on the other hand immune animals may have a comparatively low agglutination titer. In other words, with this organism the height of the agglutination titer does

not indicate the degree of immunity. As this bacillus so closely resembles the typhoid bacillus biologically and pathologically, it seems safe to conclude, until evidence is brought forth to the contrary, that in man the height of the agglutination titer does not indicate the actual degree of immunity to the latter organism. The same would apply to other members of the typhoid-colon group. It would not be wise to draw a more general conclusion until other organisms have been tested. This does not mean that agglutinins are not related to immunity but it brings up the question of the wisdom of using them as a guide in immunization with the colon-typhoid group.

When injected into the normal, vaccinated, or immune rabbit, the virulent hog-cholera bacillus is rapidly clumped and disappears from the circulation. 40 minutes after injection these organisms can be found in phagocytes in the liver. The fact that the normal rabbit gives this *intra vitam* agglutination is an exception to the findings of Bull that virulent organisms remain in the circulation for some time after injection.

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# THE NEUTRALIZATION OF ANTIPNEUMOCOCCUS IMMUNE BODIES BY INFECTED EXUDATES AND SERA.

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That sterile filtered inflammatory exudates have the power to modify the course of infection was noted long ago. The chief discussion concerning the nature of this phenomenon has been carried on by Bail and his associates and by those who have contravened their views. Bail<sup>1</sup> gave to the hypothetical substances existing in pathological exudates, which alter the course of infection, the name "aggressins." He thought that they were secreted by the bacteria during their growth in the animal body and acted by inhibiting or by neutralizing the defensive mechanisms of the body. According to this investigator it is to these substances that the bacteria owe their power to invade the body tissues and therefore it is upon their ability to form them that the property of virulence depends.

Wassermann and Citron<sup>2</sup> have opposed the view of Bail that these so called aggressins represent substances set free by the bacteria during their struggle against the protective agencies of the body, and believe that they represent merely bacterial substances which may go into solution either within the body or during growth or autolysis *in vitro*, and that these substances act by fixing the humoral immune bodies and so rendering them ineffective; that the mode of action of these substances therefore does not differ from that of dead bacteria. It is not german to the present discussion to consider the large amount of evidence which has been brought forward to support the contending views, the chief purpose of the present paper being to record the demonstration of large amounts of substances which neutralize immunity principles in the blood and exudates of animals infected with pneumococci, and to indicate the importance of these substances in the specific therapy of acute lobar pneumonia.

The part which these substances play in experimental pneumococcus infections, especially their effect on phagocytosis, has been studied by Hoke,<sup>3</sup> Rose-

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<sup>1</sup> Bail, O., *Arch. Hyg.*, 1905, lii, 272.

<sup>2</sup> Wassermann, A., and Citron, J., *Deutsch. med. Woch.*, 1905, xxxi, 1101.

<sup>3</sup> Hoke, E., *Wien. klin. Woch.*, 1905, xviii, 348.

now,<sup>4</sup> Tschistowich and Jurewich,<sup>5</sup> Zade,<sup>6</sup> and Nunokawa.<sup>7</sup> Tschistowich and Jurewich have made the observation that thoroughly washing virulent, non-phagocytatable pneumococci in salt solution is sufficient to render them phagocytatable. Rosenow, as well as Tschistowich and Jurewich, has also observed that treating non-virulent, phagocytatable pneumococci with the washings from, or extracts of virulent pneumococci is sufficient to render the former non-phagocytatable and therefore virulent, and he has found that this property is retained even after washing in salt solution. This writer has given to the hypothetical substances which may be extracted from virulent pneumococci the name "virulin," and Tschistowich and Jurewich have given to the substances which they have obtained by somewhat similar methods the name "anti-phagin."

It is evident that these observations, especially those of Rosenow, would render necessary an entirely different conception of the phenomenon from that held by Wassermann and Citron.

In a considerable number of experiments, however, I have been unable to confirm the observations of Rosenow that non-virulent pneumococci may absorb and fix something derived from virulent pneumococci which renders the former virulent, and for the present, therefore, I am inclined to accept the explanation offered by Wassermann and Citron, especially since this conception is sufficient to explain all the following observations.

The first observations on which this communication is based were made on the fluid removed from the chests of persons suffering from empyema. The fluid from these cases was examined for its content in pneumococcus immune bodies as tested by agglutination and protection. Similar tests of the patient's blood showed that it possessed well marked protective and agglutinative properties, and we were therefore surprised when we found that the empyema fluid possessed no such powers. A probable explanation seemed to be that, although the immune bodies were originally present in the exudate, they had been absorbed by the bacteria present, just as they may be from immune blood serum when bacteria are added *in vitro*. It occurred to us, however, to test this empyema fluid after removal of bacteria, to determine whether or not there might be present soluble substances,

<sup>4</sup> Rosenow, E. C., *J. Infect. Dis.*, 1907, iv, 285.

<sup>5</sup> Tschistowich, N., and Jurewich, Y., *Ann. Inst. Pasteur*, 1908, xxii, 611.

<sup>6</sup> Zade, M., *Z. Immunitätsforsch., Orig.*, 1909, ii, 81.

<sup>7</sup> Nunokawa, K., *Z. Immunitätsforsch., Orig.*, 1909, iii, 172.

which would fix or divert the immune substances contained in immune serum. The following is a protocol of one experiment.

*Case I.*—E. R.; age 19 years. Acute lobar pneumonia followed by empyema due to *Pneumococcus* Type I. 200 cc. of thick pus were removed at operation. A portion of the fluid was centrifugalized at high speed for 30 minutes, the super-

Agglutination Tests			
0.4cc Serum I + 0.4cc. empyema fluid		0.1cc Cult. I	2 hrs. 37°-18 hrs. ice
" "	(1:10) " "	"	++
" "	(1:20) " "	"	++
" "	(1:40) " "	"	+
" "	(1:50) " "	"	-
" "	(1:100) " "	"	-
" "	(1:200) " "	"	-
" " (1:10) NaCl " " (1:20) " " " (1:40) " " " (1:50) " " " (1:100) " " " (1:200) "		30 min at 37° C.	++
			++
			++
			++
			++
			++
_____ Empyema fluid undiluted		"	-
_____ " " 1:20		"	-
_____ " " 1:40		"	-
_____ " " 1:100		"	-

TEXT-FIG. 1. Protocol of an experiment showing the inhibiting action of empyema fluid on the agglutination of pneumococci by immune serum.

natant fluid was removed to a fresh centrifuge tube and again centrifugalized for 1 hour, and finally diluted with an equal quantity of isotonic saline solution and again centrifugalized for 30 minutes. The perfectly clear fluid as examined microscopically contained no organisms.

This fluid was then tested for its power to cause agglutination of Type I pneumococci and also for its power to inhibit the agglutination of pneumococci by Type I immune serum. The results are given in Text-fig. 1.

The fluid was then tested for its power to inhibit the protective action of immune serum against infection with Type I pneumococci as tested in mice. To guard against the possibility that the fluid, centrifugalized as noted above, might contain an occasional pneumococcus which might interfere with the result, it was heated for 30 minutes at 56°C. Cultures made from this fluid were sterile.<sup>8</sup> Text-fig. 2 gives the results of this experiment. They show in a striking way that empyema fluids may contain large amounts of soluble substances which inhibit the action of immune serum.

Protection Tests					
Culture dilution cc.	0.2cc. i.h.s. + 0.2cc. empyema fluid	0.2cc. i.h.s. 0.2cc. n.h.s.	0.2cc. NaCl + 0.2cc. empyema fluid	Culture control	Empyema fluid alone cc.
0.1	D. 20 hrs.	S.	D. 30 hrs.	—	0.5 S
0.01	" 21 "	"	" 16 "	—	0.3 "
0.001	" 30 "	"	" 38 "	—	0.2 "
0.0001	—	—	" 30 "	—	0.1 "
0.00001	—	—	" 30 "	D. 30 hrs.	
0.0000001	—	—	" 30 "	" 30 "	

TEXT-FIG. 2. Protocol of an experiment showing the inhibiting action of empyema fluid on the protection of mice by immune serum.

Similar tests have been carried out with a series of these empyema exudates removed by aspiration or at operation. The results have not been so striking in all the cases examined as those shown in the above protocol, though some degree of inhibition has been present in all infected cases. Several sterile serous fluids aspirated from the chest of pneumonia patients, however, have not exhibited this phenomenon. The degree of inhibiting action is apparently dependent upon the degree of infection and the time the infection has lasted before aspiration is performed.

These observations indicate why it is that infections in the partially immunized animal tend to be focal and why, when an animal is infected with organisms of slight virulence, the infection tends to remain localized. It is probable that as soon as bacteria begin to grow in tissue spaces these inhibiting substances appear in the in-

<sup>8</sup> In making the tests with mixtures of serum and fluid, the mixtures were allowed to incubate for 30 minutes at 37°C. before injection.



flammatory exudate, and when the fluid is not readily absorbed the substances accumulate in large amounts, so that finally, as in empyema, it is practically impossible to produce a focal immunity reaction until the focus is opened and the fluid, with its content of neutralizing substance, is removed by drainage, when the bacteria remaining are no longer protected from the natural or artificial defensive mechanisms of the body and so may be overcome. This conception agrees in the main with the view held by Bail and others, though the application of the theory has previously been made rather to the problems of virulence and infection than to those of recovery. The observations previously mentioned also indicate that favorable results can hardly be expected from the treatment of these focal infections with immune serum, either administered intravenously or injected directly into the focus itself unless the pathological exudate has previously been removed. We have made one attempt to treat a patient suffering from empyema by the direct injection of immune serum into the cavity, but without apparent effect. These observations offer the explanation for the failure. In the treatment of focal infections with immune serum, without drainage, it would be necessary to inject sufficient serum to neutralize all the inhibiting substances present, as well as the amount necessary to prevent the harmful activities of the bacteria themselves.

Our next problem was to discover whether or not the inhibiting substances appear in the blood as a result of septicemia. This was first investigated by inoculating rabbits with very large injections of pneumococcus and testing the blood removed during the height of infection for the presence of these substances. To show that this action is due to soluble substances, and not to the bacteria present, the bacteria have been removed from the serum by filtration before testing. The results of one of these experiments are given in the following protocol.

*Rabbit 1.*—Weight 1,300 gm. July 11, 1916, 12 noon. Inoculated intraperitoneally with 1 cc. of peritoneal exudate of a rabbit previously infected with Type II pneumococci. 5 p.m. Blood culture shows innumerable numbers of pneumococci.

July 12, 10 a.m. Animal very sick. Blood removed by heart puncture. Serum removed from clot and passed through a Berkefeld filter. Culture of filtered blood sterile. The filtered blood was tested for its power to inhibit the

agglutinating action of immune horse serum, Type II. Table I shows the results obtained.

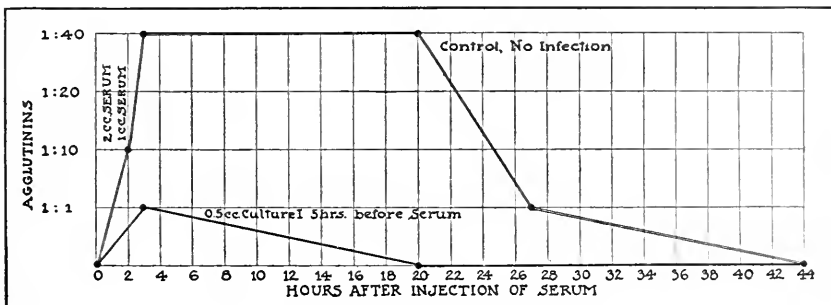
TABLE I.

Agglutination.		Results after 2 hrs. at 37°C. and 18 hrs. on ice.
0.4 cc. of Immune Serum II (1:40) + 0.4 cc. of filtered serum.....		—
0.4 cc. of Immune Serum II (1:50) + 0.4 cc. of filtered serum.....		—
0.4 cc. of Immune Serum II (1:100) + 0.4 cc. of filtered serum.....		—
0.4 cc. of Immune Serum II (1:200) + 0.4 cc. of filtered serum.....		—
	After 30 minutes at 37°C. 0.1 cc. of Culture II was added to each tube.	
0.4 cc. of Immune Serum II (1:40) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:50) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:100) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:200) + 0.4 cc. of salt solution.....		++
0.4 cc. of Immune Serum II (1:400) + 0.4 cc. of salt solution.....		+
0.8 cc. of salt solution.....		0

The results of this and other similar experiments show that specific inhibiting substances such as those which are present in pathological exudates may also be present in the blood when an animal is suffering from a severe septicemia.

Another method which has been used for testing the presence of inhibiting substances in the blood is the following. A rabbit is infected with pneumococci and after the infection has reached its height immune serum is injected intravenously. At the same time, and as a control, a normal rabbit receives the same amount of immune serum intravenously. Within a few minutes and at varying periods following the injection of the serum, samples of blood are removed from both rabbits and tested for their content in antibodies. For this purpose agglutination is employed. If no neutralization of antibodies occurs, it is evident that the content of the blood in agglu-

tinins immediately following the injection should be the same as though the immune serum had been diluted *in vitro* with a quantity of fluid equal to the blood contained in the rabbit, and that by making repeated tests a curve showing the disappearance of the immune bodies by destruction or excretion may be constructed. As a matter of fact, numerous observations in normal rabbits have shown that when the rabbit's blood is tested within a few minutes following the injection of immune serum, its content in agglutinins is about that to be expected when the probable volume of blood in the rabbit and the consequent dilution of the immune serum is calculated. On the other hand, when a similar injection is made into an infected animal,



TEXT-FIG. 3. Curves showing the agglutinating power of the serum of normal and infected rabbits following the injection of immune horse serum.

the agglutinating power of the serum obtained from it is much less than that calculated from the probable dilution; indeed, agglutinating power may be entirely absent. Moreover, when the agglutinating power is present, though lower than that of the serum of the normal rabbit, and curves are made to show the disappearance of the agglutinating power, it is found that the agglutinins disappear much more rapidly from the serum of the infected rabbit than they do from the serum of the uninfected rabbit. Text-fig. 3 shows in a graphic manner the results obtained in one of these experiments.

In these experiments the possibility cannot be excluded that the fixation or neutralization of antibodies is due to the presence of bacteria circulating in the rabbit's blood, but previous observations make it

improbable that the entire phenomenon can be due to this. It seems probable that the neutralization is due to a considerable extent to the presence of soluble inhibiting substances.

This last method of study is directly applicable to patients, and a study of this kind in patients is of importance since it is difficult to produce in animals pneumococcus infections which last over a period of a week or longer, such as those which occur in man. Moreover, it was hoped that this study would offer indications for proper dosage of serum and might even be applicable in the treatment of the individual case. In a series of cases, therefore, the serum has been tested for its content in agglutinating antibodies both before and following the administration of immune serum.

The method of procedure was as follows: Samples of the patient's serum were obtained before any immune serum was administered and also 5 minutes following the first dose. Where more than one dose was administered (and successive doses have usually been given with 6 to 8 hour intervals) other samples were obtained immediately before and 5 minutes following each subsequent dose. Finally, following the last dose, in certain cases, samples were obtained at varying periods to observe the persistence of agglutinins in the blood. The samples from each patient were kept on ice until all had been obtained and they were then tested on the same day and with the same technique for the presence of agglutinins. The agglutination tests were made by the macroscopic method. In each of a series of small test-tubes was placed 0.9 cc. of the serum, or of the diluted serum. To each of the tubes was then added 0.1 cc. of an 18 hour broth culture of pneumococcus of the type to which the infection was due and corresponding to the serum which had been injected. The results were read by transmitted light after 2 hours at 37°C. and again after the tubes had remained on ice over night.

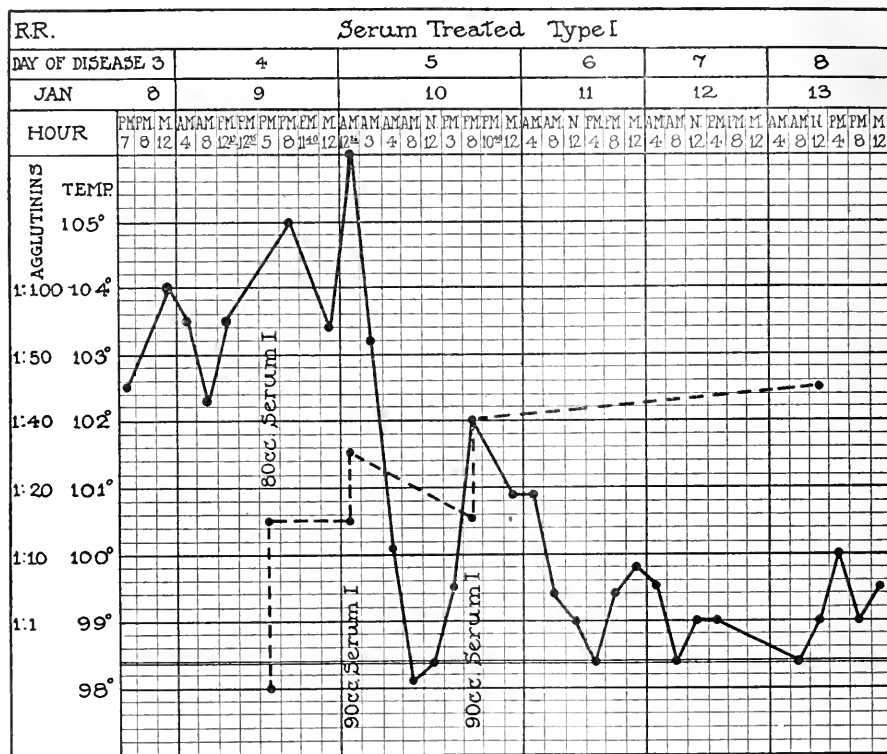
Employing these methods, agglutinin curves have been made from 30 cases suffering from Type I infection and receiving serum, 9 cases suffering from Type II infection, and also serum treated, 7 cases with Type II infection, who received no serum, 4 of Type III, not serum treated, 2 of Type IV not serum treated, and 1 case due to Type IV infection who, through a mistake in diagnosis, received several doses of Type I serum, making a total of 53 cases.

*Cases Due to Type I Infection Treated with Serum.*

Of these cases, 3 were treated on the 2nd day, 5 on the 3rd day, 5 on the 4th day, 4 on the 5th day, 9 on the 6th day, 3 on the 7th day, and 1 on the 9th day. All but two of the cases recovered. The charts and protocols of several cases, which illustrate the relation of agglutinin titer to the clinical course and to the temperature curve, are given below.

*Case 1.*—R. R., student; age 22 years. This patient was admitted January 8, 1917 at 7 p.m. suffering from pneumonia involving the left lower lobe. The onset had been quite typical with chill, 48 hours before admission. He was moderately sick; temperature 102.5°F., pulse 115, respirations 30. The leukocytes numbered 31,000 and the blood culture was positive, the plates showing one colony per cc. of blood. The sputum was bloody; a small amount was at once inoculated into a mouse. The following morning tests made of the growth in the peritoneal cavity of the mouse showed that the patient was suffering from an infection with Type I pneumococci. 12.17 p.m. The intravenous injection of antipneumococcus serum was commenced. Although the serum was given slowly, after he had received about 35 cc. he had some signs of serum intoxication, suffusion of the face, respiratory difficulty, and he vomited several times. The administration of serum was therefore at once discontinued. No tests were made of the agglutinating power of his serum before or after this treatment. The patient's condition did not materially change during the afternoon and at 5 p.m. serum was again administered; this time 80 cc. were given without any untoward symptoms. A sample of blood was taken just before and another one 5 minutes following the administration of the serum. When tested later, it was found that the blood before administering the serum contained no agglutinins for pneumococcus; the sample of blood taken following the administration of serum agglutinated Type I pneumococcus in a dilution of 1:15. This represents a concentration of antibodies fully equal to that which might be expected, taking into consideration the titer of the serum injected and the probable volume of the patient's blood. The patient's condition did not materially improve after this injection, so that another dose of 90 cc. of serum was administered at 12 midnight. A specimen of blood which was obtained just before this injection showed that the agglutinating power had not diminished during the time intervening since the preceding dose, and the specimen of blood taken 5 minutes after the serum was injected showed an increased concentration of agglutinins, so that now agglutination occurred with a 1:30 dilution of serum. Immediately following this injection the temperature rose to 106°F. and he had a shaking chill. The temperature then began to fall, being only 98.1°F. at 8 a.m. With this fall in temperature, the patient's condition markedly improved. During the day, the temperature again rose slowly, without, however, any other unfavorable features. As the temperature at 8 p.m. was

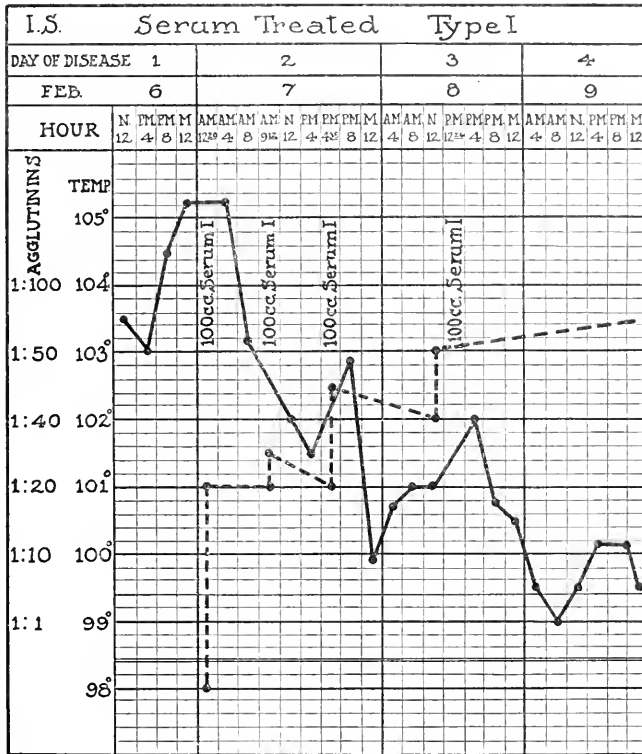
102°F., it was decided to administer another dose of serum, and 90 cc. were given, without any reaction. The test of the patient's serum obtained before this treatment showed that the agglutinins had fallen slightly, agglutination occurring in a dilution of 1:15, but after the treatment the titer again rose to 1:40. Following this treatment the patient made a good recovery.



TEXT-FIG. 4. Chart showing the curve of the agglutinin titer and the temperature curve of R. R.

In this case, therefore, which was mild or of only moderate severity, treated early in the disease, the administration of immune serum was followed by a satisfactory concentration of antibodies in the blood and there was no evidence of fixation or neutralization of the injected immune substances. This is graphically shown in Text-fig. 4.

Case 2.—I. S., tailor; age 32 years. This patient was admitted about 8 hours following the initial chill. There were definite signs of involvement of both lower lobes and he presented all the characteristic features of acute lobar pneumonia. His temperature on admission was 103.5°F., pulse 120, respirations 48. He appeared seriously sick; the blood culture taken on admission was positive. The diagnosis of the type of infecting organism was made by inoculation of sputum



TEXT-FIG. 5. Chart showing the curve of the agglutinin titer and the temperature curve of I. S.

into a mouse, and at 12 midnight, 12 hours after admission, he was given his first treatment. As the curves presented in Text-fig. 5 show, the first dose of serum imparted to the blood a well marked power of agglutination, and each subsequent dose was followed by an increase of this property. With the increase of agglutinins in the blood there occurred an improvement in the patient's condition, the temperature fell, and he finally recovered completely.

The two examples given above illustrate the effects of serum treatment in the cases due to Type I infection, when the serum is given early and in large amounts, and when the infection has not reached too high a grade before the treatments are commenced. Since in twenty-eight out of the thirty cases recovery followed the administration of the serum, we did not have great opportunity to study cases of this type in which the serum was not effective. In all the cases treated with this type of serum agglutinins could be demonstrated in the patient's blood 2 to 3 minutes after the administration of 75 to 100 cc. of serum. The agglutinating power varied somewhat, though in most cases it occurred with a dilution of 1:10 or more. The refinements of the method are not sufficient to justify our calculating in each case the probable dilution and the consequent probable loss in agglutinins in the short interval elapsing before the first tests were made. In general, where the administration of subsequent doses of the serum has not led to a prompt increase of the agglutinins in the blood above the previous level the fall of temperature has been longer delayed and more serum has been required than in the cases in which a regular step-like rise took place. In comparing the temperature and agglutination curves in these cases it has been necessary to keep in mind the fact that the temperature alone does not offer a safe and sure criterion for judging the patient's condition and therefore for the effectiveness of the serum. In view of this fact it has been surprising to see the considerable uniformity with which the temperature and agglutination curves run in opposite directions. With rise of agglutinating power the temperature curve falls.

Of more importance than the immediate rise in agglutinins following the first dose is probably the persistence of the agglutinins in the blood during the subsequent 8 to 10 hours elapsing before the following dose of serum is given. In only five of the cases did a decrease during this period occur. In three of the cases the loss occurred only following the first dose. Following the subsequent doses the concentration reached a high level and persisted. The data in these three cases is not sufficient to enable us to state categorically that this loss indicated a greater severity of infection, though taken in connection with our other observations this seems probable. Two of these three cases were treated on the 4th day and one on the 6th. They



required two, three, and four doses of serum respectively and all made good recoveries following the serum treatment. In one of the other cases in which the agglutinating power disappeared before the following dose was given, the concentration of immune bodies following the first three doses was such that agglutination did not occur with dilutions greater than 1:5, and following the first two doses this power disappeared completely before the subsequent dose was given. It was only after numerous doses had been given that the concentration of agglutinins reached any considerable level and persisted. Altogether this patient required eleven doses of serum given over 7 days. Treatment was commenced in this patient on the 2nd day, but it was not pushed with great vigor at the start, the first dose being 80 cc., with 18 hours elapsing before the administration of the second dose of 70 cc., and 12 hours again elapsing before the administration of the third dose of 80 cc. This case suggested very strongly the inadvisability of inactive treatment at the start. This patient ultimately recovered and there occurred no extension of the lesion to other lobes, but the temperature remained high for 10 days and he was very ill. Finally, the last case in which the agglutinins disappeared between subsequent doses and in which there occurred difficulty in causing a persistent concentration of immune bodies in the patient's blood by the administration of immune serum was one of the two cases which ended fatally. The curves taken from the record of this case are shown in Text-fig. 6. It is apparent from the curves that it was not until treatment had been continued for 3 days, and nine doses had been given, that a persistent concentration of immune bodies at a high level was attained. Even following this there was a constant tendency for the concentration of immune bodies in the serum to fall, rather than to rise.

It should be noted that the treatment in this patient was commenced only on the 6th day, and 11 and 13 hours elapsed between the first and second, and the second and third doses, respectively. He was desperately ill on admission; temperature 104.5°F., pulse 136, and the blood culture showed an extremely high grade of infection, over 300 colonies per cc. In spite of this he lived until the 12th day. It seems that in this case the serum prolonged life. The infection and intoxication, however, at the start were so great that, although the



infection could be kept down, the intoxication could not be recovered from. In this instance it is likely, judging from the experimental observations, that the presence of large amounts of soluble inhibiting substances in the blood prevented the action of the immune serum. It is probable that in such cases very late in the disease these substances may be so large in amount that no practical amount of immune substances can neutralize them. If these conceptions are correct, the importance of giving very large doses of immune serum at the beginning of treatment is apparent.

In the other fatal case, persistent high concentration of immune bodies in the patient's blood was obtained without difficulty. Nevertheless, the patient's condition did not improve and repeated doses of serum were administered. Type I pneumococci had been obtained from the sputum, and the blood culture showed 47 colonies per cc. of the same organisms. Treatment was commenced on the 6th day and the patient died on the 10th day. The pathological changes were extensive in both lungs. The autopsy showed a very widespread tuberculous involvement of both upper lobes and the upper portion of the lower lobe on each side. At the base of one lung, however, was a small area of complete consolidation, differing in appearance from the remainder of the tuberculous lung. This proved on study to be a typical acute diffuse pneumonic process and from it Type I pneumococci were cultivated. We have here an instance in which the serum was apparently effective against the specific infection, but death occurred on account of factors associated with the primary and extensive tuberculosis.

A further interesting case in this connection was one due to Type IV infection. Owing to a mistake in the early determination of the type of infection the patient received several doses of Type I serum before the mistake was discovered. In this patient, although he was quite ill, the administration of the serum caused a prompt appearance of agglutinins in the blood and this increased with the subsequent doses, without any material fall.

These studies of agglutination curves in the cases of Type I infection, however, while instructive and suggestive, do not after all give definite proof that the effect of immune serum is limited by the presence of soluble substances in the blood. When they are con-

sidered, however, in the light of the observations on the Type II cases which follow, the evidence becomes much more suggestive.

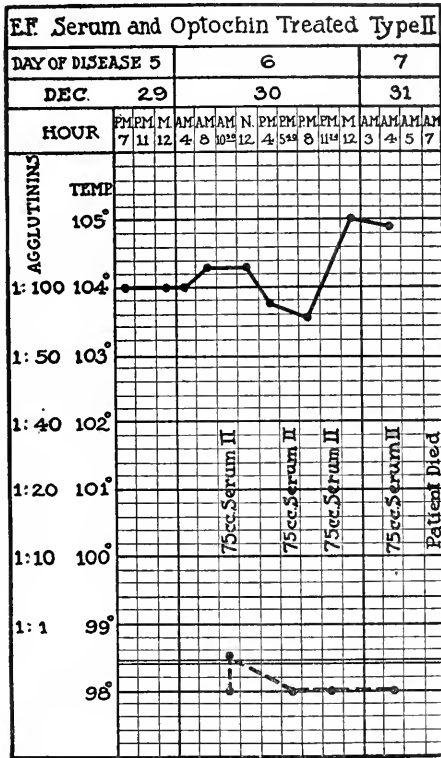
*Cases Due to Type II Infection.*

The reader should be reminded at the outset that it has been impossible to produce a serum against Type II pneumococci which is as active either *in vitro* or *in vivo* as is the serum against Type I pneumococci. Whereas the horse serum against Type I infection is of such a strength that 0.2 cc. will regularly protect a mouse against 0.1 cc. of virulent culture, it has been impossible to produce an immune Type II serum of any greater activity than that 0.2 cc. will protect a mouse against 0.01 cc. of culture. Moreover, the active Type I sera usually cause agglutination of homologous organisms in dilutions of 1:400 or over; the Type II sera usually cause agglutination in dilutions no greater than 1:200. It should also be noted that the capsule formation of Type II pneumococci is more highly developed than is that of Type I pneumococci. Dochez and Avery<sup>9</sup> have pointed out that production of precipitable substances in the blood and urine of infected animals apparently bears some relationship to this property of capsule development, the Type III organisms, which possess large capsules, forming most of this substance, the Type II organisms, which have smaller capsules, producing less, and the Type I organisms, which have small capsules, producing still less. While it is not certain that the substances in the infected animals which give rise to fixation of antibodies are identical with those concerned in the precipitation phenomenon, it seems likely that this is the case.

Studies of the agglutinin content of the blood were made in nine cases of Type II infection which received Type II serum. Of these patients, four recovered and five died. Of the patients who recovered, in two treatment was commenced on the 3rd day, in one on the 4th day, and in one on the 5th day. Of the fatal cases, treatment was commenced in one on the 3rd day, in one on the 5th day, and in the others on the 6th day. In all the four cases which recovered, a satisfactory and persistent concentration of agglutinins in the blood appeared. In one of the fatal cases, practically no agglutinins ap-

<sup>9</sup> Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.

peared in the blood in spite of four doses of serum; in one a satisfactory concentration appeared only after three doses, and then disappeared; in another a satisfactory concentration was obtained only on the 9th day, after five doses of serum; in another, while a satisfactory concentration of agglutinins was obtained on the day



TEXT-FIG. 7. Chart showing the curve of the agglutinin titer and the temperature curve of E. F.

treatment was commenced, the 6th day, the patient was at that time suffering from meningitis, from which he died 2 days later. In the remaining case only one dose of serum was given, 2 hours before death; no agglutinating power appeared in the blood. Text-figs. 7 and 8 show graphically the results of observations made in two of the fatal cases.



first dose of serum was given, the culture showed 1,600 colonies per cc. Following the first two doses of serum there occurred an immediate appearance of agglutinins in the blood in low dilutions, which, however, disappeared or became minimum in amount before the succeeding doses. In spite of the extreme grade of blood infection, the number of organisms present in the blood diminished following these two doses, the cultures on the morning of the 4th day showing only 20 colonies per cc. There was a satisfactory increase in agglutinins following the third dose, but it will be noted that 15 hours were allowed to elapse between this dose and the succeeding one, and during this time the agglutinins had entirely disappeared and the subsequent doses produced little or no effect on the agglutinin content (Text-fig. 8). The patient died on the 6th day.

Five patients suffering from Type II infection who received no serum were also studied to observe the appearance of agglutinins in the blood. All these recovered. In four of these instances at the end of the disease there developed well marked power of agglutination; in one of them agglutination in the serum obtained on the 10th day occurred in a dilution of 1:100. In the fifth no agglutinins appeared in the blood, though this was not studied later than the 12th day. This patient received optochin, as did, however, several of the cases in which agglutinins developed.

#### DISCUSSION.

Neufeld and Haendel,<sup>10</sup> Dochez,<sup>11</sup> and others have shown that specific immune substances usually appear in the blood during recovery from lobar pneumonia. This is shown by an increase in protective power of the blood for mice against homologous infection. Clough<sup>12</sup> has made similar observations and he and others have also noted that in certain instances the protective power is accompanied by the power of inducing *in vitro* phagocytosis of virulent homologous pneumococci which are not phagocytatable in normal serum. It would seem, therefore, that bacteriotropins represent one form of immune body playing a part in this protective phenomenon. Bull<sup>13</sup> has brought forward experimental evidence which indicates strongly that the phenomenon of agglutination is of great importance in the

<sup>10</sup> Neufeld, F., and Haendel, *Arb. k. Gsndtsamte.*, 1910, xxxiv, 166.

<sup>11</sup> Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.

<sup>12</sup> Clough, P. W., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 295.

<sup>13</sup> Bull, C. G., *J. Exp. Med.*, 1915, xxii, 457.

action of immune serum in pneumococcus infection. It is possible that several different antibodies or phenomena take part in the mechanism of pneumococcus humoral immunity. The observations I have mentioned, as well as unpublished observations made in this laboratory, indicate strongly that natural recovery in pneumonia is associated with the development of humoral immunity and probably occurs because of this development. In the individual case, however, the factors which determine recovery or death cannot be stated so simply. In mild cases probably a very slight grade of humoral immunity may be sufficient to prevent progress of the disease, a grade of immunity which can be detected with difficulty by our present means. In other instances the reaction required on the part of the body may be very great and the immunity phenomena exhibited by the serum when tested outside the body may be very vigorous and marked.

The phenomenon of agglutination offers one ready means for testing the degree of humoral immunity. It is, however, not the only one and it is unsafe to judge of the immunological effectiveness of a serum solely by its agglutinating strength. The protective power and agglutinating power of immune horse serum, however, tend to run parallel. Consequently, the study of agglutinating power of the blood of patients, such as has been made in the present instance, must be of considerable value in indicating the presence or absence of humoral immunity. If recovery in pneumonia is due to the development of humoral immunity, the study of its appearance during recovery and especially of its appearance following treatment with immune serum, should be of significance. In commencing the study it was thought that the method might be employed to graduate the dosage of immune serum in the treatment of the individual case. If recovery is due to the appearance of immune bodies in the blood, the ideal serum treatment would be such that sufficient serum be administered to produce the required concentration of immune bodies and no more. It soon became apparent, however, that such a method, testing the blood before and after the administration of each dose, involved so much time and labor that it would not be of practical value. It has seemed, however, that the repeated tests of the serum in a series of cases, as has been done here, give us considerable



knowledge of the mode of action of the serum and offer valuable suggestions for the routine dosage and mode of application of the serum. The studies have further indicated strongly that during infection not only must sufficient immune substances be added to bring about a concentration sufficient to sensitize all the bacteria, produce their agglutination, opsonification, etc., but in addition there must be a sufficient amount administered to neutralize any soluble substances present in the serum which have the property of neutralizing and fixing the immune substances. It is realized that the occurrence of these soluble, fixing substances in the blood of infected patients has not been directly demonstrated. The experimental observations in animals previously described, however, make it altogether probable that these substances are present in severe infections. It must be admitted that in most instances where there was failure of immune substances to appear in the blood, or where the immune bodies disappeared very rapidly following their administration, bacteriemia was shown to be present before the first dose of serum was administered. In several cases, however, the blood infection could not be demonstrated after the first dose, and nevertheless, rapid disappearance of the immune bodies occurred following the subsequent doses. In one instance in which the rapid disappearance of immune bodies occurred, the blood cultures taken both before and after the administration of serum were sterile. However, it seems likely that in all cases when fixation of immune bodies occurs, blood infection has at some time been present, though the possibility that the fixing substances may, in certain instances, arise entirely in local foci cannot be excluded.

The nature of the substances bringing about the fixation can at present only be conjectured. The demonstration, however, by Dochez and Avery<sup>9</sup> of substances giving rise to precipitates in the blood and urine of infected patients makes it probable that the same substances are responsible for the phenomenon of fixation that we have studied. They have apparently shown that these substances may be excreted or formed by the bacteria during their growth, and it is also probable that substances contained in the bacteria and set free during their dissolution may give rise to the same phenomenon.

The observations made in this study have a practical bearing on the

question of the therapeutic administration of immune serum. The amount of serum necessary to be given does not depend merely on the weight of the patient and therefore on the consequent dilution of the serum in the body. It is also not entirely dependent on the degree of infection present. If the patient is treated early before large amounts of the soluble substance are present, a moderate amount of serum may be sufficient, even though the grade of blood infection may be considerable. On the other hand, if the infection has continued for a considerable time, and large amounts of soluble, fixing substance are present in the blood, the amount of serum required may be very large. It is therefore evident that it is important that the patient be treated as early as possible and before large amounts of these fixing substances are formed. Moreover, the importance of treating very actively at the start in order that all these fixing substances may be at once neutralized and the progress of the infection immediately and entirely overcome is apparent. It is therefore our plan at present to treat all patients with Type I infection with large initial doses, and to repeat the treatment every 6 to 8 hours as long as may be necessary. It is possible that the Type II serum is less effective than Type I serum not only because its concentration of immune bodies is less than that of Type I serum, but also because the power of pneumococci of this type to produce fixing substances is more highly developed than is that of pneumococci of Type I.

#### CONCLUSIONS.

1. In empyema fluids resulting from infection with pneumococci there are present large amounts of soluble substances which have the property of neutralizing pneumococcus antibodies.
2. Similar substances are found in the blood of infected rabbits.
3. When immune serum is injected into infected rabbits the immune substances disappear very quickly, and therefore are prevented from activity in overcoming the infection.
4. When immune serum is administered to patients severely infected with pneumococci, the immune bodies may also disappear very rapidly, and this disappearance is probably associated with the presence of such soluble substances in the blood.

5. The serum only becomes effective when these substances are neutralized.

6. The study of agglutination curves is of value in showing why in certain instances favorable results have not followed the use of immune serum.

7. It is important that in severely infected patients the serum be administered early in the disease and that the initial dosage be large.



## THE ELABORATION OF SPECIFIC SOLUBLE SUBSTANCE BY PNEUMOCOCCUS DURING GROWTH.

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From the study of bacterial infections in man and the lower animals evidence has been accumulated that pathogenic organisms do not produce harmful effects from their mere physical presence. The general reaction or toxemia of infection is differentiated from the local process which in many instances is the only tangible expression of bacterial invasion. Substances of a harmful nature seem to pass out from the bacteria and through the circulating medium of the animal to injure cells and organs at a distance from the site of infection. An explanation of the nature and mode of action of these substances has been one of the great problems in the study of infection. Certain bacteria, such as diphtheria, tetanus, and others, when grown in artificial media form a soluble toxin whose action when injected into animals differs in no way from that manifested when the same bacteria grow in living tissue. The majority of pathogenic bacteria are not known to form these soluble toxins during their life processes. The capacity of the latter to intoxicate has been explained by their setting free upon death a toxin which during life is retained within the cell body. Some investigators hold the view that intoxication by these bacteria arises in the body of the infected animal from the splitting of the bacterial protein into toxic degradation products. These explanations of bacterial intoxication are not so satisfactory nor so well substantiated as are the facts concerning infection with bacteria which produce known soluble toxins.

Pneumococcus is a highly pathogenic microorganism which is not known to secrete a soluble toxin, and whose harmful effects are supposed to be due either to the setting free of intracellular toxins or

to the formation on disintegration of toxic split products. In the present paper it is shown that this organism during the early stages of its growth forms a readily soluble substance which diffuses into the culture medium *in vitro*, and in human and animal infections is present in the circulating blood, whence it passes through the kidneys into the urine. We have not as yet been able to demonstrate with certainty that this substance is responsible for the intoxication that accompanies lobar pneumonia.

### *Formation of a Soluble Substance in Culture Media.*

In 1897 Kraus<sup>1</sup> demonstrated for the first time the presence of specific precipitable substances in the germ-free culture filtrates of certain bacterial species. This work was subsequently extended by other observers so that it is now known that a large number of bacteria give rise to these substances in the media in which they grow. The precipitin reaction obtained with these substances is strictly specific and occurs only when an homologous immune serum is used. These culture fluids have been studied after the bacteria have grown in them for 24 hours or more and their presence has been supposed to be due to the passing into solution of the bacterial substance upon disintegration of the cells. The same precipitable substances may be demonstrated in the bacteria-free salt solution or distilled water extracts of organisms grown on solid media.

Neufeld<sup>2</sup> has shown that solutions of pneumococcus obtained by the addition of small amounts of bile to bouillon cultures produce a specific precipitate in the presence of immune rabbit serum. Wadsworth<sup>3</sup> has obtained similar results not only with bile solutions but also with filtered salt solution extracts of pneumococcus. Panichi<sup>4</sup> demonstrated the presence of a specific precipitable substance in the filtrate of bouillon cultures of pneumococcus.

The fact which we wish to emphasize in this study is that pneumococcus from the time it starts to grow elaborates in the medium of its environment a specific substance of bacterial origin in considerable amounts and that the early presence of this soluble substance is not attributable to the death and subsequent disintegration of the bacterial cell, but represents the extrusion into the medium of bacterial substance during the life processes of the organism.

<sup>1</sup> Kraus, R., *Wien. klin. Woch.*, 1897, x, 736.

<sup>2</sup> Neufeld, F., *Z. Hyg. u. Infektionskrankh.*, 1902, xl, 54.

<sup>3</sup> Wadsworth, A., *J. Med. Research*, 1903-04, x, 228.

<sup>4</sup> Panichi, L., *Centr. Bakteriöl., 1te Abt., Orig.*, 1907, xliii, 188.

TABLE I.  
*Rate of Growth of Culture.*

No. of hrs.	Colonies per cc.
0	810,000
2	1,170,000
4	26,000,000
6	245,000,000
8	297,000,000
12	382,000,000
24	No growth from 0.0001 cc.
48	" " " 0.1 "

*Titration of Precipitin Reaction in Culture Fluid.*

Dilution.	After 0 hrs.	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 12 hrs.	After 24 hrs.	After 48 hrs.
1:1	—*	±	+++					
1:5	—	—	++					
1:10	—	—	±	+++	+++	+++	+++	+++
1:15	—	—	±	+++	+++	+++	+++	+++
1:20	—	—	±	+++	+++	+++	+++	+++
1:25	—	—	±	++	++	+++	+++	+++
1:30	—	—	±	+	++	++	+++	++
1:40	—	—	—	+	+	++	++	+
1:50	—	—	—	±	+	+	+	+
1:60	—	—	—	±	±	±	±	±
1:80	—	—	—	±	±	±	±	±
1:100	—	—	—	—	—	±	±	±
1:120	—	—	—	—	—	±	±	±
1:160	—	—	—	—	—	—	—	±
1:200	—	—	—	—	—	—	—	±
1:240	—	—	—	—	—	—	—	±
Control.	—	—	—	—	—	—	—	—

\* — indicates no reaction; ±, faint trace; +, visible precipitation; ++, flocculation; +++, heavy flocculation.

In the following experiment a flask of bouillon was incubated with a small amount of an early, rapidly growing culture of pneumococcus. A young culture was chosen in order to avoid the occurrence of bacterial lag, during which some cell death occurs. At varying intervals during the growth of the culture, fractions were withdrawn from the flask, freed from bacteria, and the cell-free fluid was tested for the

presence of precipitable substances. A bacterial count was made of each specimen in order to determine that the culture was growing at a maximum rate and that little or no cell disintegration had occurred at the time when this substance was already present in considerable amounts. The quantity of precipitable substance present in a given specimen was determined by ascertaining the maximum dilution of the cell-free fluid at which precipitation occurred on the addition of homologous antipneumococcus serum.

A protocol in which Type III pneumococcus was studied is given, since this organism forms a large amount of soluble substance, whereas Type II and Type I form lesser amounts in the order named.

The bacterial counts in the experiment given (Table I) show that the cultures grew at a maximum rate for about 12 hours. Chesney<sup>5</sup> has shown in an elaborate study that during this period the bacteria increase in geometric progression and that the curve of generation time may be plotted as an ascending straight line. From this the deduction may be drawn that during the first 12 hours little or no cell death occurs. Examination of the precipitin reaction with the bacteria-free filtrates of specimens removed from the culture during the first 12 hours of growth reveals the fact that the bacterial substance passes into solution in the culture medium in easily demonstrable amounts during this time. This would seem to indicate that this soluble substance is not the result of bacterial disintegration but represents an actual extrusion of the cell substance into the medium during the life processes of the organism.

*Presence of Soluble Substance Derived from Pneumococcus in the Blood and Urine of Infected Rabbits.*

The demonstration that pneumococcus during its growth in fluid media gives rise to a soluble substance suggested the likelihood that the same substance might be detected in the body fluids of experimentally infected animals. In order to test this assumption, a rabbit was injected intraperitoneally with 1 cc. of the blood of a rabbit infected with pneumococcus. At varying intervals after infection specimens of blood were collected from the heart, and the serum,

<sup>5</sup> Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.



freed from cells, was passed through a Berkefeld filter in order to remove the organisms that had reached the blood stream. The bacteria-free serum was then tested for the presence of precipitable substances by the addition of homologous immune serum. The urine of these rabbits was also tested to find out whether the soluble bacterial substance passed through the kidneys and could be demonstrated by the precipitin reaction in the animal's urine. In Table II an example is given in which the rabbit had been infected with a Type II pneumococcus.

TABLE II.

*Pneumococcus Precipitin Reaction in the Blood Serum of a Rabbit Infected with Pneumococcus Type II.*

Time.	Before infection.		2 hrs. after infection.		4 hrs. after infection.		6 hrs. after infection.		8 hrs. after infection.	
	I	II	I	II	I	II	I	II	I	II
Type serum....	I	II	I	II	I	II	I	II	I	II
Result.....	—*	—	—	±	—	+ ±	—	++	—	++

\* — indicates negative; ±, faint trace; ++, marked flocculation.

Tests of rabbit urine cannot be made at regular intervals because of the failure of the animal to void frequently. Specimens at the end of 24 hours, however, showed a marked precipitate when mixed with the serum corresponding in type with the organism with which the animal was infected.

The experiment given in Table II demonstrates the fact that within a short period of time after intraperitoneal injection of a rabbit with pneumococcus there is present in the filtered blood serum a specifically reacting bacterial substance of pneumococcus origin. This substance readily passes from the blood through the kidneys into the urine and can there be demonstrated in considerable concentration.

*Presence of Soluble Substance Derived from Pneumococcus in the Blood and Urine of Patients Suffering from Lobar Pneumonia.*

The fact that the pneumococcus forms a readily soluble substance during growth in artificial media and in the body fluids of animals

experimentally infected makes it not unreasonable to assume that the same substance is formed by pneumococcus during the course of natural infection in human beings. In order to find out whether or not this is so, the blood sera and urine of a large number of patients suffering from pneumonia due to pneumococcus of Types I, II, and III were studied for the presence of this soluble material. Specimens of serum were obtained at varying intervals during the disease and the urine was examined frequently throughout the course of the infection and during convalescence. If the precipitin reaction was not positive with the whole urine, a method of concentrating the urine was employed. It has been found that the soluble substance to which the pneumococcus gives rise is precipitated by alcohol and after precipitation is again readily soluble in water. In order to concentrate the precipitable substance in urine to 25 cc. or more of the 24 hour specimen a few drops of acetic acid are added and the urine is then boiled down to a volume of 5 cc., filtered through paper to remove any precipitate of albumin that may occur, and the filtrate added to eight to ten volumes of 95 per cent alcohol. The precipitate which forms is collected by centrifugalization and dried to remove the excess of alcohol and the residue extracted with 2 or 3 cc. of salt solution which redissolves the specific substance. Any insoluble material is removed by centrifuging and the clear salt solution extract used in the precipitin test. Results of this study are presented in Table III.

In Table III are presented the studies on the presence of the specifically precipitable substance in the blood serum during life of a number of patients suffering from lobar pneumonia. Almost all the patients studied showed a strongly positive precipitin reaction in the urine and were chosen for the purpose of finding out whether at a time when the substance was being excreted in largest amounts it could also be demonstrated in the circulating blood. In all, 25 cases were examined, of which 10 were due to infection with Type I pneumococcus, 11 with Type II, and 4 with Type III. Of the Type I infections, none gave a positive precipitin reaction in the serum, although in all but one the urine was positive at the time the tests were made and in three instances the reaction in the urine was heavy, indicating the excretion of the soluble substance in considerable

TABLE III.

*Pneumococcus Precipitin Reaction in the Blood Serum during Lobar Pneumonia.*

Case No.	Blood test.		Blood culture.	Urine.	Serum treatment.	Result of disease.	Remarks.
	Day of disease.	Result.					
Pneumococcus Type I infection.							
2,821	6	—	—	++	+	Recovered.	Total, 10 cases; 2 died.
2,816	8, 9, 10, 11, 12	—	+	++	+	Died.	
2,901	5, 7, 9	—	+	++	+	"	
2,968	6, 7	—	+	+	+	Recovered.	
2,936	7, 9	—	—	±	+	"	
2,815	4	—	—	+	+	"	
2,824	3	—	—	±	+	"	
2,883	5, 6, 7	—	—	—	+	"	
2,858	3, 4, 5	—	—	+	+	"	
2,891	3	—	—	+	+	"	
Pneumococcus Type II infection.							
2,885	3, 4, 6	—	—	+	+	Recovered.	Blood culture positive on 6th day.
2,845	6	++	+	++	+	Died.	
2,868	4	—	—	—	—	Recovered.	
	7			+			
2,829	1	—	—	—	—	"	
2,879	2	—	+	—	+	"	
	7			+			
2,892	5, 6	+	+	++	—	Died.	
2,834	6, 7, 9, 10, 11	—	+	++	+	"	
2,922	4, 5, 6	—	—3	+	+	Recovered.	
			+7				
3,006	2, 3	—	—	++	—	Died.	
3,031	3, 5, 7	+	+	++	+	"	
2,869	3, 4, 5	+	±	+	+	"	
Pneumococcus Type III infection.							
2,898	5, 6, 7	++	+	++	—	Died.	Total, 4 cases.
2,947	6, 10, 11	±	—	+	—	Recovered.	
2,797		+	—	+	—	Died.	
2,783	7	+	+	+	—	"	

quantity. The failure to demonstrate the substance in blood in Type I pneumonias may be partly attributable to the fact that all these cases were treated with Type I antipneumococcus serum, which is known to cause the disappearance of the substance from the urine in many cases during treatment. It has also been shown by *in vitro* experiments that Pneumococcus Type I forms less of the soluble substance than organisms of Types II and III. The sera of 11 cases of Type II pneumonia were studied. The urine reaction was positive in 10 of these, while the precipitin reaction in the blood was positive in 4 instances. Of these 11 cases 7 were treated with Type II serum. Among the 7 serum treated cases, 3 showed a positive precipitin reaction in the blood. Of the 4 cases not treated with serum, only 1 gave a positive blood test. A positive blood culture was obtained in 7 of the 11 cases studied. Of the 4 cases with a positive precipitin test in the serum, all showed a positive blood culture, while of the 7 cases with a negative precipitin test in the serum, 3 had a positive blood culture. All 4 patients showing a positive precipitin reaction in the blood serum died, whereas of the 7 with a negative serum test, 2 died and 5 recovered.

4 cases of pneumonia due to Pneumococcus Type III were studied. All gave a positive precipitin test in the blood serum. Blood cultures were positive in 2 instances and the precipitin reaction in the urine was positive in all. The infection was fatal in 3 of the 4 patients.

In Table IV are presented the results of the examination of the urine for the precipitable pneumococcus substance in 88 cases of pneumonia due to the fixed types of pneumococcus I, II, and III. Of these 88 cases, 35 were Type I, 28 were Type II, 8 were Type II (atypical), and 17 were Type III. Repeated tests of the urine were made during the course of the disease from within 12 hours after onset in one instance to the 58th day in another. Of the 35 cases due to Type I infection, 20 were positive and 15 negative. A positive blood culture occurred in 13 of the 35 cases. Among the 13 cases with positive blood culture, 9 showed a positive urine reaction and 4 gave a negative result. Of the 20 cases with positive urine reaction, 2 died; of the 14 negative cases all recovered. All these instances of Type I infection were serum treated. The administration of serum

TABLE IV.

*Pneumococcus Precipitin Reaction in the Urine during Lobar Pneumonia.*

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type I infection.								
				days				
2,816	++	6	6	6	+	+	Died.	
2,936	+	6	6	18	-	+	Recovered.	
2,968	++	5	5	14	+	+	"	
2,858	+	3	4	1	-	+	"	
2,996	+	3	3	15	-	+	"	
2,891	+	3	3	5	-	+	"	
2,965	+	6	6	16	-	-	"	
2,952	+	2	5	3	-	+	"	
2,804	+	6	6	26	-	+	"	
2,955	++	6	6	26	+	+	"	
2,925	+	1	1	1	+	+	"	
2,908	+	5	13	5	-	+	"	
2,924	+	5	5	22	-	+	"	
2,913	+	5	5	1	-	+	"	
2,945	+	5	5	29	+	+	"	
2,906	+	2	2	31	+	+	"	Delayed resolution.
2,852	+	4	4	2	+	+	"	
2,949	+	8	11	24	-	+	"	
2,901	+	5	5	4	+	+	Died.	
2,917	-	6	-	-	-	+	Recovered.	
2,814	-	2	-	-	-	+	"	
2,944	-	5	-	-	-	+	"	
2,874	-	3	-	-	+	+	"	
2,815	-	4	-	-	-	+	"	
2,824	-	3	-	-	-	+	"	
2,821	-	6	-	-	-	+	"	
2,984	-	7	-	-	-	+	"	
3,020	-	3	-	-	+	+	"	
3,043	-	3	-	-	-	+	"	
3,011	-	5	-	-	+	+	"	
2,883	-	4	-	-	-	+	"	
2,954	-	4	-	-	-	+	"	
2,880	-	3	-	-	+	+	"	
3,019	+	4	8	42	+	+	"	
3,033	-	2	-	-	-	+	(empyema). Recovered.	

TABLE IV—*Continued.*

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type II infection.								
				<i>days</i>				
2,869	++	2	2	2	+	+	Died.	
2,922	+	3	3	40	+	+	Recovered.	
3,006	+	2	2	8	—	—	Died.	
2,885	+	2	2	2	—	+	Recovered.	
2,845	++	5	5	2	+	+	Died.	
2,868	+	4	7	3	—	—	Recovered.	
2,899	—	1	—	—	—	—	"	
2,879	—	2	7	1	+	+	"	
2,892	++	5	5	2	+	—	Died.	
2,834	++	5	5	5	+	+	"	
2,991	+	1	2	58	—	—	Recovered.	
2,854	—	3	5	1	—	—	"	
3,003	+	7	7	13	—	—	"	
2,827	++	3	3	1	+	—	Died.	
2,746	+	3	6	9	+	—	"	
2,881	+	6	6	2	—	—	Recovered.	
2,786	—	6	—	—	—	—	"	
2,896	—	7	—	—	—	—	"	
2,926	—	4	—	—	—	—	"	
2,897	+	3	4	1	—	+	"	
2,886	+	3	3	6	—	—	"	
2,890	+	3	3	4	+	+	Died.	
2,971	+	7	14	10	—	—	Recovered.	
2,825	++	1	1	5	+	—	Died.	
3,031	+	3	3	5	+	+	"	
3,047	+	3	3	?	—	—	Recovered.	
2,937	—	1	—	—	—	—	"	
2,934	—	4	—	—	—	—	"	
Pneumococcus Type II (atypical) infection.								
2,861a	—	3	—	—	+	—	Recovered.	
2,878x	—	4	—	—	—	—	"	
2,822b	—	5	—	—	—	—	"	
2,831x	+	4	4	3	—	+	"	
2,864b	+	8	8	2	+	—	Died.	
2,960x	—	1	—	—	—	—	"	
2,963b	+	4	7	13	—	—	Recovered.	
2,935b	—	1	—	—	—	—	"	

TABLE IV—*Concluded.*

Case No.	Urine reaction.	Day of disease when urine was first tested.	Day of disease when urine was first positive.	Duration of positive reaction.	Blood culture.	Serum treatment.	Result of disease.	Remarks.
Pneumococcus Type III infection.								
2,838	—	3	—	—	—	—	Recovered.	
2,889	—	7	—	—	—	—	"	
2,797	++	2	2	5	—	—	Died.	
2,898	++	5	5	3	+	—	"	
2,947	++	5	5	30	—	—	Recovered (em-pyema).	
2,812	+	2	5	1	+	—	Died.	
2,783	+	7	7	1	+	—	"	
2,919	+	5	6	4	—	—	"	
2,800	+	3	3	3	+	—	"	
2,849	+	3	3	2	+	—	"	
2,927	+	2	6	4	—	—	Recovered.	
2,973	+	3	10	4	—	—	"	
2,911	+	2	2	13	—	—	"	
2,837	—	1	—	—	—	—	"	
2,972	+	1	6	2	—	—	"	
2,485	—	6	—	—	+	—	Died.	
2,918	—	2	—	—	—	—	Recovered.	

*Summary of Urine Reaction.*

Type.	Total No. of cases.			Per cent positive.	Positive blood culture.	Positive urine reaction.		Negative urine reaction.		Fatal cases showing urine reaction.	
	Examined.	Positive.	Negative.			With blood culture positive.	With blood culture negative.	With blood culture positive.	With blood culture negative.	Positive.	Negative.
I	35	20	15	57.1	13	9	11	4	11	2	0
II	28	20	8	71.4	11	10	10	1	7	10	0
II (atypical)	8	3	5	37.5	2	1	2	1	4	1	1
III	17	12	5	70.5	6	5	7	1	4	7	1
	88	55	33	62.5	32	25	30	7	26	20	2
IV	10	0	10	0	2	0	0	2	8	0	2
Cases of respiratory disease due to organisms not pneumococcus.	14	0	14	0	2	0	0	2	12	0	2

in Type I pneumonia often results in the temporary disappearance of the substance from the urine. Upon cessation of treatment the soluble substance may reappear in the urine.

Of 28 cases of Type II infection, 20 gave positive precipitin reaction in the urine, and 8 were negative. A positive blood culture occurred in 11 of the 28 cases. Among the 11 cases with positive blood culture, 10 gave a positive urine reaction and 1 a negative result. Of the 20 cases with a positive urine test, 10 died; of 8 negative cases all recovered.

Among 8 cases of infection with atypical Type II pneumococcus, 3 gave a positive precipitin reaction in the urine and 5 a negative reaction. Inasmuch as a normal Antipneumococcus Serum Type II was used in determining the presence of soluble substance in the urine of individuals infected with atypical Type II pneumococcus, a lower percentage of positive urine reactions should be expected in this series, since the precipitin titer of normal Type II serum is low for these atypical organisms.

17 cases of pneumonia due to infection with *Pneumococcus* Type III were studied. *Pneumococcus* precipitinogen was demonstrated in the urine of 12 of these instances, and was absent in 5. A positive blood culture was obtained in 6 of the 17 cases. Of the 6 cases having pneumococcus septicemia, 5 showed a positive precipitin reaction in the urine. 7 of the 12 cases giving a positive urine test died, while 4 of the 5 negative cases recovered.

A summary of 88 cases of pneumonia due to the fixed types of pneumococcus shows that the soluble substance of pneumococcus origin was demonstrable in the urine of 55 (62.5 per cent) of these patients at some stage of the disease and in 39 instances was positive on the first examination. Among the 55 cases with a positive precipitin reaction in the urine, 20 had a fatal outcome, giving a mortality of 36.4 per cent, and of the 33 cases with a negative urine test, 2 died; a mortality of 6 per cent. In addition to the 88 individuals suffering from pneumonia due to the fixed Types I, II, and III, 10 cases of Type IV pneumonia and 14 cases of respiratory disease due to other organisms were studied for the presence of a precipitin reaction in the urine. Each urine was tested with standard Antipneumococcus Sera Types I, II, and III. In no instance was a posi-



tive reaction obtained at any stage of the disease, a fact which establishes beyond doubt the specificity of the reaction.

In addition to the presence of the soluble substance of pneumococcus origin in the blood and urine, it has also been found in other body fluids. In certain cases it can be readily demonstrated in pleural fluids and pericardial exudates and in the spinal fluid of pneumococcus meningitis.

Certain facts have been ascertained concerning the chemical characteristics of this substance. The specific substance is not destroyed by boiling. It is readily soluble in water and is precipitable in acetone, alcohol, and ether, after which it may be easily redissolved in water. It is precipitated by colloidal iron, and does not dialyze through parchment. The immunological reactions of the substance are not affected by proteolytic digestion with trypsin and it is not split by urease. The determination of total nitrogen and nitrogen partition on the active substance obtained by repeated precipitation with acetone and alcohol shows the substance to be of protein nature or to be associated with protein.

One of the chief points of interest in the discovery of the soluble substance of pneumococcus is whether this substance is in any way responsible for the intoxication which attends pneumococcus infection. Studies to ascertain the answer to this question are being actively carried on at the present time but have not as yet progressed to the point at which a definite answer can be given. It may be said, however, that its toxicity is in no way comparable to that of diphtheria toxin. On the other hand, it possesses a degree of toxicity which, exhibited throughout the course of an infection, may account for the signs of intoxication in lobar pneumonia.

#### DISCUSSION.

The preceding experimental data have shown that a specifically reacting substance of pneumococcus origin occurs in the bacteria-free filtrates of young cultures of pneumococcus and also in the blood serum and urine of patients during lobar pneumonia. The occurrence of specifically precipitable substances in the cell-free filtrate of bacterial cultures has been known ever since the early obser-

vation of Kraus. It has been abundantly confirmed by other investigators with a variety of bacteria. In general, the presence of this precipitable substance has been demonstrated in culture fluids so old that an opportunity has occurred for cell death and disintegration and consequent solution of bacterial protein. In this paper it is shown that there is present in solution in the culture fluid in which pneumococcus is grown, a soluble substance in considerable amounts at a time when no cell death or disintegration has occurred. Consequently this substance does not represent dead dissolved bacterial protein, but the elaboration and passage into solution of a substance which is the product of the life activity of the cell. In addition to the evidence already cited in support of this fact, it has been demonstrated that the soluble substance is present in culture fluids in considerable concentration at a time when no hemolysin is present. This pneumococcus hemolysin is an intracellular body which does not appear in culture fluids until destruction of the bacterial cell has taken place; hence if the soluble substance described were purely of intracellular origin the curve of its concentration in culture fluids would be coincident with that of the hemolysin. This, however, is not the case, for the curve of hemolysin does not begin to rise until a time when the curve of the soluble substance has almost attained its maximum elevation.

The formation of a soluble substance by the pneumococcus on growth *in vitro* suggested the probability that an analogous substance would be formed on growth of the organism in the animal body and because of the readiness with which the substance passes into solution one would expect no difficulty in demonstrating it in the body fluids of experimentally infected animals. Examination of the blood and urine of rabbits infected with pneumococcus has shown this substance to be present in considerable quantities following intraperitoneal infection. Ascoli and Valenti<sup>6</sup> have demonstrated in the organisms of animals infected with anthrax a substance specifically precipitable with antianthrax serum. Bail<sup>7</sup> has shown the presence of a substance in the exudates of animals infected with anthrax which, when the fluids were freed from bacteria, increased the infectious

<sup>6</sup> Ascoli, A., and Valenti, E., *Centr. Bakteriolog., 1te Abt., Ref.*, 1911, xlviii, 243.

<sup>7</sup> Bail, O., *Arch. Hyg.*, 1905, lii, 272; 1905, liii, 302.

power of anthrax bacilli. This substance he has called aggressin and he considers it to be an excretory product of the anthrax bacillus which favors the invasion of animal tissues by this organism. It is possible that the substances described by Ascoli and Valenti, and Bail are similar in the mechanism of their formation to the soluble substance produced by the pneumococcus. Although our study of other bacteria has been rather limited, it has been demonstrated that certain other species, such as meningococcus, *Bacillus typhosus*, and *Bacillus dysenteriae*, also give rise to soluble material during their growth in fluid media.

A study of the serum of patients suffering from lobar pneumonia has shown that this soluble specific substance is also present in the circulating blood during the course of the disease in man. It gives a specific precipitin reaction with antipneumococcus serum corresponding in type to the organism with which the individual is infected. This soluble precipitable substance in human serum is less frequently present in demonstrable quantities than in the serum of experimentally infected animals. However, it has been found both when pneumococci are present in the circulating blood and when by blood culture organisms are absent. Complement fixation, as well as the precipitin reaction, may be used for the demonstration of this substance in serum. Although the soluble substance is relatively infrequently present in demonstrable quantities in the circulating blood, it is not unlikely, from the fact that the substance appears in a much larger percentage of cases in the urine, that it is much more commonly present in the blood than observed, but in quantities that are below the threshold of demonstration.

A study of the urine in 112 cases of lobar pneumonia and closely related respiratory diseases has shown that in 62.5 per cent of pneumonia due to Pneumococcus Types I, II, and III, a substance is excreted in the urine which reacts specifically with antipneumococcus serum of the type corresponding to the organism with which the individual is infected. This substance may appear as early as 12 hours after the initial chill, or may appear for the first time at a later stage of the disease, and may continue to be excreted for many days after recovery has occurred. In certain instances in which excretion occurred over a long time, its persistence in the urine could

be explained by delayed resolution, a condition which represents the passage of the acute pneumococcus infection of the lung into one of a more chronic character. In other instances of continued excretion not explainable on these grounds, the substance must have been stored in the tissues and must have passed into the circulating blood to be excreted by the kidneys without loss of its specific character. It is the rule to find the substance in the urine when pneumococcal septicemia exists. The amount of precipitable substance in the urine seems to be a measure of the severity of the infection. This fact may be dependent upon the quantity of the substance being directly proportional to the actual amount of infection or it may be that the amount of this substance formed bears some relationship to the virulence of the particular strain of pneumococcus responsible for the infection. Most of the instances which fail to show the presence of a precipitable substance in the urine recover, whereas the mortality is high among those in which its presence is demonstrable. If large amounts are excreted the outcome is usually fatal, unless this result is prevented by the administration of anti-pneumococcus serum. The specific precipitin test in the urine is therefore of considerable prognostic value. It may also be used in making a rapid diagnosis of the type of organism with which an individual is infected and in our experience a positive test in the urine is quite as reliable as the agglutination of the organism isolated from the sputum. The precipitin test in the urine, however, should not supplant the usual diagnostic technique in the determination of the type of pneumococcus.

11 years ago Fornet<sup>8</sup> claimed to have demonstrated in the serum and urine of patients suffering from typhoid fever a substance specifically precipitated by antityphoid serum. From what we now know it would seem likely that his observations were correct despite the fact that subsequent investigators failed to confirm them.

Ascoli<sup>9</sup> has shown that precipitinogen may pass the kidneys and appear in the urine where it exhibits its specific reaction. We have been able to show that if rabbits are inoculated intravenously with

<sup>8</sup> Fornet, O., *Münch. med. Woch.*, 1906, xxxviii, 1862.

<sup>9</sup> Ascoli, M., cited in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, ii, 750.

soluble pneumococcus material a specific precipitin reaction can be obtained in the urine within 24 hours and that the specific substance continues to be excreted for a number of days. In such an experiment the material injected contained no formed living pneumococci. Pettit<sup>10</sup> has demonstrated that if rats are injected with diphtheria toxin, this substance can be shown to be excreted in the urine in active form following the inoculation.

#### SUMMARY.

1. A specifically reacting substance of bacterial origin is present in the cell-free fluids of young cultures of pneumococcus. This substance is present when the organisms are growing at their maximum rate and undergoing little or no cell death, and consequently its presence is not dependent upon cell disintegration but represents the extrusion of bacterial substance by the living organism.

2. The blood and urine of rabbits experimentally infected with pneumococcus contain a similar specific soluble substance during the early hours of the infectious process.

3. Human beings suffering from lobar pneumonia have in their blood and more frequently in their urine a specific soluble substance of pneumococcus origin. The amount of this substance present in the urine varies in different individuals and the presence of a large amount is of unfavorable prognostic import. This specific precipitin reaction in the urine is of diagnostic value.

4. Rabbits injected with soluble pneumococcus material continue to excrete this substance for a considerable period of time.

5. The specifically soluble substance obtained from bacterial cultures and from the urine during infection is not destroyed by boiling, by precipitation with alcohol, acetone, or ether, or by trypsin digestion.

6. Studies are in progress at this time on the degree of toxicity and on the antigenic properties of the substance.

<sup>10</sup> Pettit, A., *Ann. Inst. Pasteur*, 1914, xxviii, 663.



## ACIDOSIS AND ACID EXCRETION IN PNEUMONIA.\*

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During the course of certain studies of the various factors of acid excretion,<sup>1</sup> 24 hour samples of urine from several normal and pathological subjects which were examined demonstrated that, in the absence of  $\beta$ -hydroxybutyric acid, the hydrogen ion concentration and titratable acidity are due largely to the ratio between acid and basic phosphates. Although in the main this hypothesis was quite well supported in the twenty-three samples analyzed, in one fatal case of pneumonia, over 60 per cent of the titratable acid could not be accounted for by the phosphates. This observation led to a more extended investigation of acidosis and acid excretion in acute lobar pneumonia, the results of which are presented in this paper.

There exists certain evidence which supports the idea that the metabolism during the febrile stage of pneumonia results in the production of considerable amounts of acid substances. Increased ammonia and titratable acid in the urine have been observed. Diminished carbon dioxide in the blood has been reported by several observers, notably by Peabody<sup>2</sup> who reviews the literature of the subject. Recently Lewis<sup>3</sup> has found the blood of patients with pneumonia to have a decreased affinity for oxygen, a characteristic of acidosis. Palmer and Henderson<sup>4</sup> noticed that during the fastigium of the disease larger amounts of sodium bicarbonate by mouth were necessary to reduce the acidity of the urine than is normally the case. Such facts as these, increased ammonia and acid excretion, low carbon dioxide in the blood, diminished affinity of the blood for oxygen, and retention of large amounts of alkali, indicate an excessive acid production during the febrile stage of the disease.

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\* A brief report of this work was given before the American Society for Clinical Investigation, Atlantic City, N. J., May 7, 1917.

<sup>1</sup> Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1914, xvii, 305; 1915, xxi, 37; *Arch. Int. Med.*, 1915, xvi, 109.

<sup>2</sup> Peabody, F. W., *J. Exp. Med.*, 1912, xvi, 701.

<sup>3</sup> Lewis, T., *Lectures on the Heart*, New York, 1915.

<sup>4</sup> Palmer and Henderson, *Arch. Int. Med.*, 1913, xii, 153.

### *Methods.*

The several factors determined in the urine were the hydrogen ion concentration by the colorimetric method previously described,<sup>5</sup> titratable acidity by the method to be described presently, ammonia by Folin and Macallum's method,<sup>6</sup> and the total phosphates by the uranium acetate method. The combined carbon dioxide in the plasma was determined by the Van Slyke-Stillman-Cullen method.<sup>7</sup> As the method of titrating the urine has been modified for a special purpose and, so far as known, has not been used before, it will be described in detail. In the earlier part of the work the method as described by Henderson and Palmer<sup>1</sup> was used, but on account of the variation in the hydrogen ion concentration of the urine and because of the especial interest in the fluctuation in the difference between the total titratable acidity and the phosphate acidity of the urine, it seemed desirable to determine the titratable acidity between two fixed reactions, or in other words to estimate the quantity of acid or base involved in a specified change of hydrogen ion concentration at a level which is a function of the acids in question. By doing this it is possible to compare these differences at a fixed hydrogen ion concentration.

The hydrogen ion concentration<sup>8</sup> of 5.0 was selected as the acid end-point because most acid urines in pneumonia, while not infrequently reaching this degree of acidity, seldom exceed it. The hydrogen ion concentration of 7.4 as the neutral point was for obvious reasons retained as in the titration previously described. Two titrations are necessary and are carried out as follows: 10 cc. of a 0.1 molal solution of monopotassium phosphate, 4 parts, and disodium phosphate, 25 parts

<sup>5</sup> Henderson and Palmer, *J. Biol. Chem.*, 1912-13, xiii, 393.

<sup>6</sup> Folin, O., and Macallum, A. B., *J. Biol. Chem.*, 1912, xi, 523.

<sup>7</sup> Van Slyke, D. D., Stillman, E., and Cullen, G. E., *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 165.

<sup>8</sup> Throughout this paper the hydrogen ion concentration has been expressed by the negative exponent of the hydrogen ion concentration, that is, Sørensen's pH. The minus sign is omitted for convenience; for example, a hydrogen ion concentration of 5 means a pH of 5 or an  $H^+$  of  $10^{-5}$ . It should be remembered that an actual increase in hydrogen ion concentration is indicated by a decrease in this exponential figure.



(yielding a hydrogen ion concentration of 7.4) are introduced into a 250 cc. flask, diluted to 250 cc. with distilled water, and 0.2<sup>9</sup> cc. of a 1 per cent aqueous solution of neutral red is added. 10 cc. of urine are similarly diluted, neutral red is added, and 0.1 N sodium hydrate is run in until the color matches that of the phosphate solution. The second part of the titration is accomplished by introducing 10 cc. of a 0.14 molal solution of acetic acid, 23 parts, and sodium acetate, 46 parts (yielding a hydrogen ion concentration of 5.0) into a 250 cc. flask, diluted to 250 cc. with distilled water, and five drops of a 1 per cent aqueous solution of sodium alizarin sulfonate are added. 10 cc. of urine are similarly diluted, sodium alizarin sulfonate is added, and 0.1 N hydrochloric acid run in until the color matches that of the acetic acid-sodium acetate solution. In matching the color of the alizarin solutions, especially when the urine is highly colored, it has been found desirable to place a flask containing 10 cc. of urine diluted as for titration but without indicator, behind the flask containing the standard acetic acid-sodium acetate solution with indicator. Also a flask with distilled water is similarly placed back of the urinary sample which is being titrated. The titration must be carried out in front of a window with good light. The titratable acidity in 0.1 N cc. between the hydrogen ion concentration of 5.0 and the hydrogen ion concentration of 7.4 is the sum of these two titrations.

With the above data available, it is possible to compare the actual titratable acidity with that which the phosphates alone would give. The calculation of the part of the phosphates is simple. In the equilibrium<sup>10</sup>  $H^+ = K \times \frac{HA}{BA}$  approximately, where  $H^+$  is the hydrogen ion concentration,  $K$  the ionization constant  $k$  for the acid divided by  $\alpha$ , the degree of ionization of the salt of the acid,  $HA$  the undissociated acid, and  $BA$  the salt of the acid,  $H^+$  is determined and  $K$  is known, hence  $\frac{HA}{BA}$  may be easily calculated; and if the total phos-

<sup>9</sup> It has been found convenient and satisfactory to use a dropping bottle for adding the indicator. Care should always be taken to add the same size and number of drops to standard and urine samples. Three to five drops are sufficient.

<sup>10</sup> Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 427.

phates are known the amount of HA and BA can be estimated. Reference to Sørensen's measurements shows that this titration is sufficiently accurate within the ranges of hydrogen ion concentration involved in the present investigation. It is appreciated, however, that the presence of electrolytes and variation in the concentration of the urine combine to decrease the accuracy of the titration but not to a degree sufficient to alter the conclusions reached. An example of the calculation follows.

The  $\text{pH} = 5.3$  or  $\text{H}^+ = 50 \times 10^{-7}$ , titratable acidity amounts to 200 cc. 0.1 N acid, total phosphates in terms of phosphorus pentoxide is 1.0 gm. When the acid is monobasic phosphate  $K = 2.5 \times 10^{-7}$ . We have then, from the above equilibrium,  $50 \times 10^{-7} = 2.5 \times 10^{-7} \times \frac{\text{HA}}{\text{BA}}$ , whence  $\frac{\text{HA}}{\text{BA}} = \frac{20}{1}$ ; that is, 96 per cent of the phosphate exists as the monobasic phosphate. 1 gm. of phosphorus pentoxide corresponds to 141 cc. of 0.1 N monobasic phosphate. Hence in a mixture of mono- and dibasic phosphates containing 1.0 gm. of phosphorus pentoxide and having a hydrogen ion concentration of 5.3, 135 cc. exist as the acid and 6 cc. in the basic form. As our method of titrating the urine carries the reaction to a hydrogen ion concentration of 7.4 or when the ratio  $\frac{\text{MH}_2\text{PO}_4}{\text{M}_2\text{HPO}_4} = \frac{4}{25}$ , 14 per cent of the phosphate is in the acid form, then the per cent of the acid phosphate which actually takes part in the titration from a hydrogen ion concentration of 5.3 to a hydrogen ion concentration of 7.4 is  $96 - 14$  or 82 per cent. Hence 82 per cent of 141 cc. amounts to 115 cc. and the difference between the actual acidity and acidity calculated from the phosphates is  $200 - 115$  or 85 cc.

This difference is probably due largely to free organic acids and will be so designated in the discussion following. In Table I the percentage of acid phosphate for any hydrogen ion concentration between 5.0 and 7.4 has been computed, as well as the amounts of acid phosphate entering into the titration between any hydrogen ion concentration within these limits and 7.4.

TABLE I.

*Ratio of Acid, HA, to Basic, BA, Phosphate at a Hydrogen Ion Concentration Varying between 7.4 and 4.9 with the Percentage of Acid Phosphate Taking Part in Titration When Carried to a Hydrogen Ion Concentration of 7.4.  $K = 2.5 \times 10^{-7}$ .  $pH = 7.4$  Which Is the Reaction of Normal Blood.*

pH.	$H^+ \times 10^{-7}$ .	$\frac{HA}{BA}$	HA.	HA-14.
			<i>per cent</i>	<i>per cent</i>
7.4	0.4	$\frac{4}{25}$	14	0
7.2	0.6	$\frac{6}{25}$	19	5
7.0	1.0	$\frac{10}{25}$	29	15
6.9	1.3	$\frac{13}{25}$	34	20
6.8	1.6	$\frac{16}{25}$	39	25
6.7	2.0	$\frac{20}{25}$	45	31
6.6	2.5	$\frac{25}{25}$	50	36
6.5	3.2	$\frac{32}{25}$	56	42
6.4	4.0	$\frac{40}{25}$	62	48
6.3	5.0	$\frac{50}{25}$	67	53
6.2	6.3	$\frac{63}{25}$	72	58
6.1	8.0	$\frac{80}{25}$	76	62
6.0	10.0	$\frac{100}{25}$	80	66
5.9	13.0	$\frac{130}{25}$	84	70
5.8	16.0	$\frac{160}{25}$	87	73
5.7	20.0	$\frac{200}{25}$	89	75
5.6	25.0	$\frac{250}{25}$	91	77

TABLE I—*Concluded.*

pH.	$H^+ \times 10^{-7}$ .	$\frac{HA}{BA}$	HA.	HA-14.
			<i>per cent</i>	<i>per cent</i>
5.5	32.0	$\frac{320}{25}$	93	79
5.4	40.0	$\frac{400}{25}$	94	80
5.3	50.0	$\frac{500}{25}$	95	81
5.2	63.0	$\frac{630}{25}$	96	82
5.1	80.0	$\frac{800}{25}$	97	83
5.0	100.0	$\frac{1000}{25}$	98	84
4.9	120.0	$\frac{1200}{25}$	98	84

## OBSERVATIONS.

For a preliminary survey 24 hour samples of fresh urine from six normal individuals and seventeen cases representing a wide variety of pathological conditions selected from the medical wards of the Massachusetts General Hospital were examined, the results of which appear in Table II.

Case 15 stands out very prominently with 323 cc. of 0.1 N free organic acid, while of the remaining cases 10 showed less than 50 cc., 7 less than 100 cc., 4 less than 150 cc., and 1 normal individual showed as much as 187 cc. It is possible that the high value in Case 6 may be explained partly by the high hydrogen ion concentration and partly by the size of the individual, or, indeed, by a possible abnormality in metabolism. Cases 16 and 23 had high temperatures but showed only small amounts of free organic acid when compared with the total acidity. The ammonia excretion in Case 15 is the highest in the series but is not excessive.

TABLE II.  
*Normal and Pathological Individuals.*

No.	Volume of urine.	Weight.	pH.	Acid 0.1 N.	Ammonia 0.1 N.	Acid + ammonia 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Free organic acid at the determined pH,* 0.1 N.	Remarks.
	cc.	kg.		cc.	cc.	cc.	gm.	cc.	cc.	
1	1,420	94	6.0	336	450	786	2.45	228	108	Normal.
2	1,560	64	6.1	272	375	647	2.31	202	70	"
3	2,000	79	6.3	320	590	910	3.95	295	25	"
4	2,100	89	6.7	143	458	601	2.33	102	41	"
5	1,100	73	5.7	386	418	804	3.05	323	63	"
6	1,000	91	5.4	415	350	765	2.02	228	187	"
7	2,000	61	5.9	186	280	466	1.34	132	54	Chronic alcoholism.
8	740	80	6.1	126	420	546	1.55	135	-9	Arteriosclerosis.
9	1,700	68	5.7	180	293	473	1.40	148	32	Pernicious anemia.
10	2,650	69	7.0	64	382	446	2.16	46	18	Cardiorenal disease.
11	750	78	5.3	125	252	377	0.63	72	53	" "
12	1,290	44	6.0	184	160	344	1.45	135	49	Pleurisy with effusion.
13	2,440	50	6.3	176	346	522	2.02	151	25	" " "
14	2,500	51	6.0	182	390	572	1.58	147	35	Unexplained edema of lower legs.
15	2,360	70	5.3	580	750	1,330	2.25	257	323	Acute lobar pneumonia.
16	580	62	5.3	242	550	792	0.90	103	139	" endocarditis. Temperature 103°F.
17	1,540	68	5.5	510	595	1,105	3.38	377	133	Diffuse sclerosis of central nervous system.
18	1,120	66	6.3	210	392	602	1.95	146	64	Cholelithiasis.
19	1,350	74	6.2	155	141	296	1.18	97	58	Convalescent; typhoid fever.
20	560	63	5.6	145	170	315	0.88	96	49	Cholelithiasis.
21	2,500	50	6.7	100	380	480	1.20	52	48	Chronic constipation.
22	900	57	5.7	206	355	561	1.10	116	90	Diabetes (ferric chloride reaction negative).
23	1,775	60	5.8	445	683	1,128	3.24	334	111	Typhoid fever. Temperature 104.0°F.

\* All samples gave no color with ferric chloride.

*Normal Individuals.*—Four normal subjects of widely varying weights were examined. Very little difference between the individuals was found, hence only one of the protocols is given (Table III).

TABLE III.

*Factors Determined on a Normal Individual, Weight 70 Kilos, for 9 Consecutive Days.*

Volume of urine.	pH.	Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 3.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.
cc.		cc.	cc.	gm.	cc.	cc.	cc.	cc.	cc.
1,080	5.5	318	378	1.63	182	190	136	188	346
1,270	5.6	324	414	1.81	196	212	128	202	432
1,000	5.6	320	400	1.86	202	220	118	180	280
1,090	5.5	175	251	1.70	189	202	-14	49	344
600	5.5	180	246	1.00	112	118	68	128	132
1,070	6.6	257	460	1.92	96	228	161	232	293
1,930	6.4	250	425	1.52	103	180	147	245	367
1,015	6.0	142	234	0.85	79	100	63	134	238
1,510	5.7	288	378	1.72	180	202	108	176	417

There is considerable variation in the amounts of free organic acid both when calculated from the hydrogen ion concentration at which the urine was passed and when estimated at the hydrogen ion concentration of 5.0. The hydrogen ion concentration of the case selected varies very little although the free organic acid varies considerably. In general, as the hydrogen ion concentration increases in value, that is, as the urine becomes more alkaline, the amount of organic acid present in the free state is less. On the other hand, as the reaction approaches a hydrogen ion concentration of 5.0 the organic acid fraction increases very rapidly.

*Individuals with Acute Lobar Pneumonia.*—In all, thirty cases of acute lobar pneumonia, involving the analysis of 325 24 hour samples of urine, were studied. It is not necessary to report in detail all of the protocols of the series. Certain cases have been selected as representative of the various conditions found in the group (Tables IV, V, VI, VII, and VIII). In general it may be said that the more severe the intoxication the greater the amounts of free organic acid at the hydrogen ion concentration of 5.0 which are present. The type of organism<sup>11</sup> was determined in twenty-three of the cases, showing 4

<sup>11</sup> Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

with Type I, 11 with Type II, 4 with Type III, and 4 with Type IV.

One of the four with Type I infection showed an increase in the free organic acid but in small amount only. In six of the Type II group there was a very marked increase, in three a moderate increase of the free organic acid, while two were without any significant change. There was no increase in the acid excretion in any of the Type III cases. This finding is not what might be expected *a priori* because this type of infection has proved to have the highest mortality of all, 50 per cent of the cases being fatal. One of the group studied died, the protocol of whom is given in Table V. Two of the Type IV cases showed some increase in the free organic acid excretion; the others did not.

The case reported in Table IV was chosen as an example of those individuals who showed no particular signs of either acidosis or increase in acid excretion from the laboratory or clinical standpoint. The total amount of acid excreted is not excessive; although the ammonia excretion on the 6th and 7th days of the disease just before the crisis is greater than on the days following, it is not large. At no time is the combined carbon dioxide of the plasma below normal. The lower limit of normal given by Van Slyke, Stillman, and Cullen is 55 volume per cent, which corresponds to an alveolar air of about 38 mm. carbon dioxide tension. The acidity of the urine fell at the time of the crisis, and this occurred in most of the cases having a definite crisis.

The protocol of a fatal case appearing in Table V is given to illustrate the unexpected finding in the Type III infection. Although the intoxication in this infection was very great, there was no marked increase in the free organic acid nor was there any other evidence of acid intoxication. The ammonia and acid excretion, as well as the combined carbon dioxide in the plasma, were well within normal limits.

In Table VI are given the data of a case with Type II infection which is a fair example of what frequently occurs in the more severe infections.

At the time of the crisis the amounts of acid not accounted for by the phosphates fall off and quite regularly during convalescence

TABLE IV.

*Hospital No. 2,852; Male; Age 33 Years. Process Confined to the Left Lower Lobe.  
Type I Infection. Blood Culture Positive. Treated with Serum.  
A Severe Chill Followed the Fifth Treatment, after Which  
the Temperature Fell by Crisis.*

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i	Remarks.
	*F.			cc.		Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.	
5	104.5 102.5	124 108	54 46	1,060	5.4	460	533	2.50	282	296	178	237	650	58.6	175 cc. of antipneumococcus serum.
6	103.6 100.8	106 96	48 40	1,105	5.5	196	265	0.51	57	60	139	205	910	60.6	300 cc. of antipneumococcus serum. Chill.
7	106.5 98.2	154 72	46 30	1,185	5.7	250	440	1.20	127	142	123	298	975		
8	100.5 99.5	88 72	40 28	985	6.1	145	334	0.74	65	88	80	246	630	61.7	
9	99.5 99.2	76 60	40 24	865	6.6	152	372	1.10	43	130	109	242	430		
10	100.4 98.5	76 60	36 28	1,200	6.4	370	665	3.00	204	356	166	309	605		
11	99.5 98.5	78 64	28 20	900	5.8	310	436	1.80	184	212	126	224	790	62.2	
12	100.4 99.0	80 74	32 28	900	5.6	225	336	1.27	138	150	87	186	378		
13	100.4 99.4	88 80	28 20	625	5.6	73	145	0.83	90	98	-17	47	295	69.1	
14	101.6 100.0	100 80	40 20	1,230	5.6	325	450	1.68	182	198	143	252	410		Beginning of serum disease.
15*	102.6 100.5	100 80	36 20	850	5.5	278	356	1.32	147	156	131	200	283		

\* This case was followed for a week longer but as there was nothing of note in the data, they are not given.



TABLE V.

*Hospital No. 2,593; Male; Age 37 Years. Process at the Entrance of the Right Lower Lobe Extending 2 Days before Death to the Entire Right Lung.  
Type III Infection. Blood Culture Positive. Treated with  
Optochin. Died on the 9th Day of the Disease.*

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i	Remarks.
	°F.			cc.		Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.	
3	104.0 102.5	112 102	38 32	1,425	5.8	184	395	1.57	162	186	22	209	427	54.1	
4	104.0 103.0	116 100	42 36	1,675	5.7	218	400	1.71	180	202	38	198	524	59.8	
5	103.0 102.0	112 100	44 32	2,200	5.6	396	600	3.34	363	396	33	204	880		
6	103.0 102.0	112 104	40 32	2,040	5.2	388	470	3.04	352	360	36	110	885	51.3	
7	102.0 102.0	120 102	48 32	1,510	5.2	348	410	2.60	300	308	48	102	800	50.4	Died 2 days later.

keep within normal limits. Early in the investigation before the free organic acid fraction was compared at the fixed point of pH = 5.0, this remarkable decrease was somewhat misleading due to the fact that the hydrogen ion concentration of the urine changed to the nearly neutral point at the crisis. On the 7th day of the disease the free organic acid excreted in 24 hours amounted to 760 cc., while in the days following the limits were between 220 and 351 cc. There was also a marked increase in the ammonia excretion, amounting to 2,050 cc. on the 7th day, rapidly coming down to normal values

TABLE VI.

*Hospital No. 2,865; Male; Age 23 Years. Process Involving the Entire Left Lung.  
Type II Infection. Blood Culture Negative. Treated with Optochin.  
Recovery.*

Day of disease.	Maximum and minimum temperature.		Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.				cc.		Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pent-oxide.	Phosphorus pent-oxide acid 0.1 N, pH 7.4.	Phosphorus pent-oxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
4	106.0 105.4	120 110	48 32		1,740	5.2	588	640	1.47	170	174	418	466	432	62.5
5	104.8 103.4	116 104	48 40		2,350	5.0	910	910	4.00	474	474	436	436	595	61.4
6	103.0 101.0	100 84	44 32		3,510	5.8	990	1,380	7.00	723	828	267	552	1,580	65.3
7	100.5 99.2	102 72	48 30		2,300	6.2	660	1,120	3.04	248	360	412	760	2,050	66.8
8	101.2 100.0	76 70	40 28		1,555	6.5	202	507	1.32	78	156	124	351	1,130	60.8
10	101.2 99.8	80 46	30 24		925	5.8	390	565	2.42	250	287	140	278	694	63.8
11	101.0 98.0	76 60	32 24		650	5.6	387	500	2.36	256	280	131	220	700	
12	99.5 99.0	68 58	24 20		900	5.8	400	532	2.63	271	312	129	220	510	
13	99.8 98.3	72 60	24 20		925	5.5	500	563	2.75	306	325	194	238	495	
14	99.2 98.6	64 60	22 20		1,688	5.4	450	515	2.10	236	248	214	267	580	
15*	99.5 99.0	96 60	22 20		1,200	5.6	412	460	2.03	220	240	192	220	327	

\* This case was followed a week longer but as the data contained nothing of especial interest they are not given.

after the crisis. A decided fall in urinary acidity occurred after the crisis. Throughout the entire course of the disease, however, the combined carbon dioxide in the plasma remained normal, indicating the ability of the organism to cope with the increased acid production.

The case reported in Table VII excreted the largest amount of free organic acid in 24 hours of any of the cases observed. On the 4th day of the disease 1,165 cc. of the total acidity of 1,800 cc. at a hydrogen ion concentration of 5.0 were unaccounted for by the phosphates. The intoxication was intense. That the mechanism for regulating the acid-base equilibrium was sufficient is proved by the combined carbon dioxide in the plasma. Only 2 hours before death it was 62.5 volume per cent, the lowest at any time during the disease.

TABLE VII.

*Hospital No. 2,869; Male; Age 31 Years. Process in the Right Lower Lobe. Type II Infection. Blood Culture Positive. Treated with Serum and Optochin. Died.*

Day of disease.	Maximum and minimum temperature.		Maximum and minimum pulse.		Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.							Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
3*	103.4	120	48		cc.	cc.										
	103.0	108	32	4,340	5.5	1,600	1,900	9.65	1,070	1,140	530	760	737	64.1		
4*	103.3	124	48													
	102.4	108	30	3,160	5.6	1,380	1,800	5.35	580	635	800	1,165	1,730	64.1		
5*†	104.0	128	52													
	102.0	114	30	1,900	5.9	475	820	2.88	284	340	191	480	688	63.5		

\* 190 cc. of antipneumococcus serum.

† Died on the 6th day of the disease. The combined carbon dioxide in the plasma 2 hours before death was 62.5 volume per cent.

TABLE VIII.

*Hospital No. 2,634; Male; Age 42 Years. Process Confined to the Right Lower Lobe.  
Type IV Infection. Blood Culture Negative. Treated with Optochin.  
Recovery.*

Day of disease.	Maximum and minimum temperature.	Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.	a	b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.			cc.		Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pentoxide.	Phosphorus pentoxide acid 0.1 N, pH 7.4.	Phosphorus pentoxide acid 0.1 N, pH 3.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
7	104.8	106	44											
	103.4	90	32	1,975	5.3	623	730	4.10	470	486	153	244	1,040	60.7
8	104.6	96	48											
	103.0	82	30	1,580	5.4	350	440	2.24	252	266	98	174	1,060	60.7
9	104.0	84	46											
	102.5	82	32	1,775	5.2	370	497	2.62	304	310	66	187	1,200	52.8
10	103.5	88	40											
	100.0	72	32	2,560	5.7	294	460	2.62	278	310	16	150	1,800	64.5
11	102.5	70	30											
	99.0	56	24	1,550	5.7	268	392	1.83	193	216	75	176	1,180	68.3
12	99.2	72	32											
	98.6	58	24	1,415	5.5	318	410	2.20	246	261	72	149	900	
13	99.2	76	32											
	98.6	56	24	1,630	5.6	394	542	3.02	328	358	66	184	625	
14	99.6	84	26											
	98.6	58	18	1,665	5.5	372	478	2.45	273	290	99	188	500	
15	99.4	80	20											
	98.6	64	18	1,715	5.2	394	437	2.50	290	296	104	141	620	54.1
17	99.0	70	24											
	98.6	52	18	1,490	5.0	540	540	3.12	370	370	170	170	690	
18	99.6	80	18											
	98.8	58	18	1,300	5.0	430	430	2.50	296	296	134	134	533	

TABLE VIII—*Concluded.*

Day of disease.	Maximum and minimum temperature.		Maximum and minimum pulse.	Maximum and minimum respirations.	Volume of urine.	pH.		a	b	c	d	e	f (a-d)	g (b-e)	h	i
	°F.							Acid 0.1 N.	Acid pH 5.0-7.4, 0.1 N.	Phosphorus pent-oxide.	Phosphorus pent-oxide acid 0.1 N, pH 7.4.	Phosphorus pent-oxide acid 0.1 N, pH 5.0-7.4.	Free organic acid at the determined pH, 0.1 N.	Free organic acid at the pH of 5.0, 0.1 N.	Ammonia 0.1 N.	Combined carbon dioxide in plasma. Volume per cent.
20	99.6	80	20	20	1,500	5.0	480	480	2.74	325	325	155	155	540		
	98.7	60	18	18												
21	99.6	80	20	20	1,380	5.0	400	400	2.60	308	308	92	92	458		
	98.8	56	18	18												
23	100.0	80	20	20	1,080	5.0	260	260	1.67	198	198	62	62	290	49	
	98.8	64	20	20												
24	100.0	80	24	24	1,415	5.0	396	396	2.28	270	270	126	126	315		
	99.2	56	18	18												
25	100.2	72	18	18	1,140	5.0	348	348	2.32	280	280	68	68	413		
	99.2	60	18	18												
27	99.5	84	18	18	1,135	5.0	330	330	1.83	217	217	113	113	330		
	99.2	70	18	18												

The individual with a Type IV infection reported in Table VIII revealed a condition not found in any of the other cases studied. While there were only small amounts of free organic acid excreted during the fastigium of the disease and in convalescence, the ammonia excretion was very high until after the crisis, when the 24 hour values became normal. There was never any significant lowering of the combined carbon dioxide in the plasma.

## DISCUSSION.

The facts brought out by the investigation are that in many, usually the more severe, cases of acute lobar pneumonia there are excreted during the fastigium of the disease considerable quantities of an organic acid which is free at a hydrogen ion concentration of 5.0, and that

there is seldom a severe grade of acidosis as estimated by the amount of combined carbon dioxide in the plasma. The nature and biological importance of this organic acid are not without interest. Certain possibilities are suggested. Because of its prevalence in many conditions where there is abnormal metabolism,  $\beta$ -hydroxybutyric acid was searched for, although all specimens examined had a negative or at most a very faint ferric chloride reaction. The ionization constant of  $\beta$ -hydroxybutyric acid is  $2 \times 10^{-5}$ , therefore one-third of the acid is free in a urine with a hydrogen ion concentration of 5.0. Quantitative estimates revealed insignificant amounts. This is not surprising when one considers, for instance, that in Table VI on the 7th day of the disease the acid unaccounted for amounts to 760 cc. and if it were all due to  $\beta$ -hydroxybutyric acid there would be present a total of 24 gm. of the acid, an amount which is seldom encountered except in the more severe grades of acidosis in diabetes. Hippuric acid with an ionization constant of  $2.2 \times 10^{-4}$ , acetoacetic acid with an ionization constant of  $1.5 \times 10^{-4}$ , and lactic acid with an ionization constant of  $1.4 \times 10^{-4}$  whereby only 5 to 7 per cent of an acid can exist free at a hydrogen ion concentration of 5.0 could hardly be expected to account for any considerable quantities of free organic acid. Acetic acid, the ionization constant of which is  $1.9 \times 10^{-5}$ , exists about one-third free at a hydrogen ion concentration of 5.0, hence it becomes a possibility. While uric acid with an ionization constant of  $1.5 \times 10^{-6}$  may be 87 per cent free at a hydrogen ion concentration of 5.0, the total amount of this substance is easily estimated and has never been found to account for more than a few cubic centimeters of the free acid. Nor can the conjugated sulfuric acid be responsible for any large quantities as shown by several ethereal sulfate determinations. The oxy- and other less well known acids are possibilities, but as their ionization constants are not known nothing definite can be stated about them. In Table VIII the acid is apparently a fairly strong one as shown by the high ammonia without much free acid or high total phosphates.

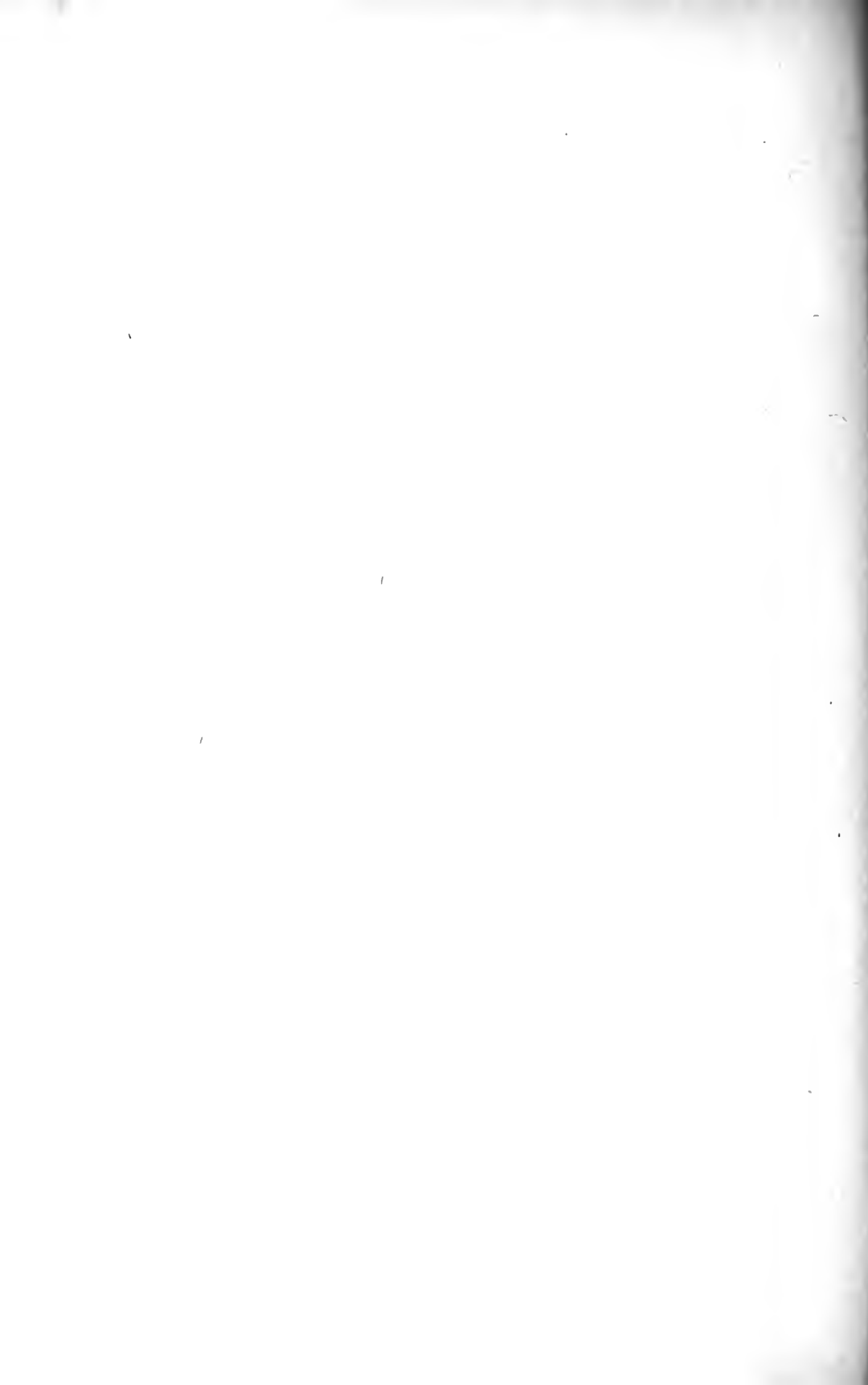
As it has been shown that normally at a hydrogen ion concentration of 5.0 there exists a certain titratable acidity that cannot be accounted for by the phosphates, it is possible that the increase in this value during pneumonia may be due simply to an increase of a normal

constituent of the urine. On the other hand, the possibility of abnormal oxidation products leads one to suspect that there may exist some pathological substances to account for the phenomenon and this in part has been borne out by our investigation. If this is the case, its part, if any, in the intoxication encountered in the disease is of much interest. Considerable investigation of the nature and significance of the increase in the free organic acid production of the urine during pneumonia has been carried out, and will form the subject of a future communication.

#### SUMMARY.

There is excreted in the urine of subjects ill with acute lobar pneumonia a large amount of organic acid which is free at a hydrogen ion concentration of 5.0.

Acidosis as determined by the combined carbon dioxide in the plasma is seldom, if ever, severe.





## FURTHER STUDIES ON THE EPIDEMIOLOGY OF LOBAR PNEUMONIA.

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Previous studies on the epidemiology of lobar pneumonia have shown that although pneumococcus is present in the mouths of about 50 per cent of normal individuals, it is extremely rare to find pneumococcus of Type I or Type II in the normal mouth except in individuals who have been in intimate association with patients suffering from lobar pneumonia due to organisms of these types. The observations of Dochez and Avery<sup>1</sup> suggest that in many instances lobar pneumonia may be due to contact infection. The source of such infection may be either an individual who has recovered from lobar pneumonia but who still harbors in his mouth the infectious microorganism, or a healthy carrier who has acquired the organism from association with a case of lobar pneumonia.

In the present paper are reported, first, the varieties of pneumococcus isolated from cases of lobar pneumonia admitted to the Hospital of The Rockefeller Institute during the past 5 years, second, the varieties of pneumococcus found in the mouths of normal individuals, third, a study of the types of pneumococcus present in the mouth secretions of members of households where cases of Type I and Type II pneumonia have occurred, fourth, the types of pneumococcus obtained from the dust of rooms in which no case of pneumonia is known to have occurred, fifth, the types of pneumococcus obtained from the dust in homes where pneumonia of Type I or Type II has occurred, and sixth, two epidemics of pneumonia.

<sup>1</sup> Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1915, xxii, 105.

*Varieties of Pneumococcus Concerned in the Production of Lobar Pneumonia.*

During the past 5 years a study has been carried on of the types of pneumococcus isolated from 454 cases of lobar pneumonia admitted to the wards of the Hospital of The Rockefeller Institute. These organisms have been isolated from sputum, from blood cultures, or directly from the lung. In many instances they have been isolated from more than one of these sources and classified in the biological group already described by Dochez and Gillespie.<sup>2</sup> The results of this classification are shown in Table I.

TABLE I.  
*Types of Pneumococcus Causing Lobar Pneumonia in 454 Cases.*

Pneumococcus.		Incidence.	
			<i>per cent</i>
Type	I.....	151	33.26
"	II.....	133	29.29
"	II a.....	6	1.32
"	II b.....	4	0.88
"	II x.....	9	1.98
"	III.....	59	12.99
"	IV.....	92	20.26

The incidence of Types II a, II b, and II x, subvarieties of Type II, described by Avery,<sup>3</sup> has only been determined during the last 2 years.

*Varieties of Pneumococcus in the Normal Mouth.*

Pneumococci were isolated in 116 instances from the saliva of 297 normal individuals in whom no history of contact with a previous case of lobar pneumonia could be obtained. In five instances two types of pneumococci were isolated from the same individual. Pneumococcus Type I was isolated only once; Pneumococcus Type II was not found among these 297 healthy individuals. Among the 39 per cent showing pneumococcus in the mouth secretions, the dis-

<sup>2</sup> Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

<sup>3</sup> Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

TABLE II.

*Types of Pneumococcus Isolated from the Saliva of Normal Individuals Not in Direct Contact with Lobar Pneumonia.*

Pneumococcus.	Incidence.	
		<i>per cent</i>
Type I.....	1	0.8
" II.....	0	0
" II a.....	1	0.8
" II b.....	7	5.8
" II x.....	14	11.6
" III.....	34	28.1
" IV.....	64	52.9
Pneumococcus present.....	116	
" absent.....	<u>181</u>	
	297	

tribution of types is in striking contrast to that which is found among individuals in direct contact with disease. In Table IV are given the results of the examinations of 184 normal individuals known to be in contact with a case of lobar pneumonia due to pneumococci of Types I and II. Among 107 normal individuals known to be intimately associated with a Type I infection, 15 per cent showed pneumococcus of the same type as that causing disease. Of the remaining 77 individuals, all of whom were in direct contact with a Type II infection, this organism was isolated in 5 instances (6 per cent). In all, pneumococci of the more strictly disease-producing types, I and II, were found in 11 per cent of individuals in direct contact with cases of lobar pneumonia due to these types; while among 297 individuals in whom no history of previous contact could be obtained, pneumococci of Types I and II were found in only 0.8 per cent.

The pneumococcus is found in the mouths of many normal individuals. In some the same type of pneumococcus is present constantly, while in others the types may vary from time to time or entirely disappear. In Table III is presented a study of the types of pneumococcus recovered from the saliva of 942 healthy persons.

From the saliva of 942 normal individuals including those in contact with lobar pneumonia one or more types of pneumococcus were

TABLE III.

*Types of Pneumococcus Isolated from the Sputum of Normal Individuals,  
Including Those in Direct Contact with Lobar Pneumonia.*

Pneumococcus.	Incidence.	
		<i>per cent</i>
Type I.....	34	7.0
" II.....	22	4.5
" II a.....	1	0.2
" II b.....	26	5.3
" II x.....	47	9.7
" III.....	85	17.5
" IV.....	271	55.8
Pneumococcus present.....	450	
" absent.....	492	
	942	

isolated in 450 instances, or 47 per cent. In 492 individuals no pneumococci could be recovered at the time of examination. Type I pneumococcus was isolated from 34 individuals. 33 of these persons had recently been in close association with a case of lobar pneumonia due to the same type. In only one instance was there no history of contact. From 22 individuals Type II pneumococcus was obtained. 19 of these persons had recently been intimately associated with a case of lobar pneumonia of Type II. It is significant that only once was pneumococcus of Type II a isolated from a normal mouth. Yet this organism was responsible for six cases of pneumonia, or 1.32 per cent. On the other hand, Pneumococcus Type II b was found 26 times and Pneumococcus Type II x 47 times in normal mouths. In view of the relative frequency of Types II b and II x in normal mouths, these organisms, as those of Types III and IV, must be considered as normal inhabitants of the healthy mouth. It is not infrequent to find two types of pneumococcus at the same time in one mouth. But as yet a Type I and a Type II pneumococcus have never been found simultaneously in the same mouth.

*Incidence of the Carrier Condition in Normal Individuals in Contact with Patients Suffering from Lobar Pneumonia.*

Dochez and Avery<sup>1</sup> have shown that many individuals who come in contact with active cases of lobar pneumonia due to infection with *Pneumococcus* Type I and Type II carry in their mouth secretions a pneumococcus of the same type as that causing the disease with which they have been associated. In order to determine the frequency with which normal individuals acquire these types of pneumococcus, the following study was carried on.

A nurse was sent to the home as soon as possible after a patient suffering from pneumonia due to Type I or Type II had been admitted to the hospital. Specimens of sputum were collected from all members of the household; *i.e.*, all persons who had come in contact with the patient. In Table IV are presented the results of this study. This table includes all the cases which were studied from this point of view. It shows the incidence and type of pneumococcus found among the members of 52 households. From 28 of these homes came patients with lobar pneumonia due to Type I infection. Among the 107 members of these families 16, or 15 per cent, showed Type I pneumococcus. One or more positive contacts were found in 10 (36 per cent) of the homes. Type II pneumococcus was isolated from only 1 individual. 77 individuals from 24 homes in which a case of Type II pneumonia had occurred were studied. From 5, or 6 per cent, a Type II pneumococcus was isolated. In 5, or 21 per cent, of the 24 homes a positive contact was found. A Type I pneumococcus was not found among these individuals. Altogether, from a total of 52 cases of lobar pneumonia due to Type I and Type II, 15, or 28 per cent, gave rise to the carrier condition in at least one of their immediate associates. The total number of households examined was 52, and in 15, or 28 per cent, there were one or more persons who showed pneumococcus of Type I or Type II in their sputum. Of the 184 persons who composed these households 21, or 11 per cent, showed either a Type I or a Type II pneumococcus.

The average carrying period for Type I was 25 days, and for Type II 43 days. The carrying period was roughly estimated by taking the middle date between the last positive sputum and the first negative one. If the contact was still positive at the last examination, a plus sign is added.

TABLE IV.

*Incidence of the Carrier Condition in Healthy Individuals in Contact with Lobar Pneumonia.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,746	I	Father. Mother. Sister T. " A.	IV No pneumococcus. IV IV	
2,751	II	Wife. Sister. Brother. Brother-in-law.	II x and III No pneumococcus. IV II b and IV	
2,786	II	Husband. Son. Daughter. Friend.	II x IV IV II	48
2,804	I	" Mr. O. " Mrs. O. " G. " H.	IV IV II x IV	
2,814	I	Wife. Brother.	IV II b	
2,815	I	Husband. Daughter R. " H.	No pneumococcus. I No pneumococcus.	6
		Friend.	II x	
2,816	I	Wife. Son.	No pneumococcus. I	31
2,821	I	Sister-in-law. Brother. Friend.	No pneumococcus. II II b	Undetermined.
2,824	I	Sister.	No pneumococcus.	
2,825	II	Wife. Son-in-law. Daughter.	II No pneumococcus. II x	48
2,827	II	Wife. Friend.	No pneumococcus. II b and III	
2,834	II	Sister. Niece T. " M. Nephew H. Friend C. " D.	IV IV IV II b and IV II b " III II b " IV	

TABLE IV—Continued.

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,852	I	Mother. Sister M. " A. Brother L. " I.	No pneumococcus. IV No pneumococcus. " " II x	Undetermined.
2,853	II	Mother. Father. Sister E. " A. Brother.	No pneumococcus. IV No pneumococcus. " " " "	
2,854	II	Wife. Daughter H. " M. " I.	" " " " " " III and IV	
2,858	I	Friend. Wife. Daughter. Son R. " I.	II No pneumococcus. IV II x and IV IV	
2,868	II	Mother. Brother E. " A. " W.	II b No pneumococcus. " " " "	
2,869	II	Wife. Daughter A. " I. Brother.	IV No pneumococcus. " " " "	
2,874	I	Friend F. " J. " H.	II x III II x	
2,879	II	Wife.	No pneumococcus.	
2,880	I	Mother. Father. Brother W. " R.	IV No pneumococcus. IV III	
2,881	II	Sister. " Brother. Brother-in-law. Niece.	I and IV IV No pneumococcus. II x and III IV	41

TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,883	I	Son. Daughter. Friend S. " Y.	No pneumococcus. " " " " II x	
2,885	II	Maid J. " A. " M.	IV No pneumococcus. " "	
2,886	II	Sister C. " E. Niece. Brother-in-law.	" " II x No pneumococcus. IV	
2,890	II	Wife.	No pneumococcus.	
2,891	I	Sister. Niece E. " M.	" " " " " "	
		Brother-in-law.	" "	
2,892	II	Wife. Daughter X. " M.	" " II x III	
		Son.	II x	
2,896	II	Boarder. Mother. Sister. Brother.	No pneumococcus. IV IV II x	
2,901	I	Wife. Friend K.	I II x	80
2,906	I	Husband. Sister. Son.	No pneumococcus. " " " "	
2,908	I	Wife. Brother. Sister L. " A.	" " IV No pneumococcus. I	
2,913	I	Mother. Brother.	No pneumococcus. IV	37
2,917	I	Mother. Father. Brother C. " B. Sister S. " N.	IV No pneumococcus. III No pneumococcus. IV No pneumococcus.	



TABLE IV—*Continued.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,922	II	Wife.	II	70+
		Daughter I.	No pneumococcus.	
2,924	I	Wife.	" "	
		Daughter K.	" "	
		" L.	I	25
		" A.	No pneumococcus.	
		Son.	IV	
2,925	I	Wife.	III	
		Daughter S.	No pneumococcus.	
		" A.	" "	
		" R.	III	
		Son J.	I	25
		" M.	III	
2,926	II	Friend M.	III	
		" F.	No pneumococcus.	
		" L.	II	7
2,934	II	" R.	No pneumococcus.	
		" N.	IV	
		" H.	II x	
		" C.	No pneumococcus.	
		" K.	" "	
2,944	I	Mother.	I	32
		Father.	II b	
		Husband.	I	28
		Friend M.	No pneumococcus.	
		" McC.	IV	
		Daughter M.	No pneumococcus.	
		" H.	I and III	16
		Son.	No pneumococcus.	
		Sister.	I and II x	17
2,945	I	Mother.	I	7
		Father.	I	35+
		Brother I.	No pneumococcus.	
		" M.	" "	
		" C.	III and IV	
		Sister E.	I	9
		" Y.	No pneumococcus.	
		" S.	" "	
2,946	II	Wife.	" "	
		Son.	IV	

TABLE IV—*Concluded.*

Case No.	Type of infecting pneumococcus.	Relationship of associates.	Type found in associates.	Duration of carrying period.
				<i>days</i>
2,952	I	Wife.	IV	
		Daughter.	No pneumococcus.	
2,954	I	Brother E.	" "	
		" J.	" "	
		Sister R.	" "	
		Cousin.	" "	
2,955	I	Wife.	I	21
		Son.	II x	
		Daughter.	I	5
2,968	I	Sister-in-law.	No pneumococcus.	
		Brother.	" "	
		Nephew J.	II b	
		" O.	IV	
2,971	II	Maid.	No pneumococcus.	
		Father.	" "	
2,976	II	Mother.	" "	
2,984	I	Brother.	IV	
		Nurse.	IV	
2,991	II	Sister.	IV	
2,996	I	Wife.	II x	
		Son.	No pneumococcus.	
M.M.	II	Wife.	IV	
		Mother-in-law.	IV	
		Nurse.	II x	

*Summary.*

Type.	No. of pneumonia households examined.	Households in which carriers were found.		Total contacts examined.	Positive contacts.	
			<i>per cent</i>			<i>per cent</i>
I.....	28	10	36	107	16	15
II.....	24	5	21	77	5	6
Total.....	52	15	28	184	21	11

*Types of Pneumococci Obtained from Dust of Households in Which No Case of Pneumonia Had Occurred.*

The presence of pneumococcus in dust has been a known fact for some time. Netter<sup>4</sup> in 1897 was the first to recover a definite pneumococcus from dust. Previous workers, notably Emmerich,<sup>5</sup> demonstrated the presence of Friedländer's bacillus in the dust of a room where there were many pneumonia patients. But at this early date the pneumococcus and Friedländer's bacillus were not clearly differentiated. The occurrence of the pneumococcus in dust has been lost sight of and very little significance has ever been attached to it. Its presence in dust has not been correlated with the occurrence of cases of pneumonia beyond a few casual references to the finding of pneumococcus in the dust of wards where there were many pneumonia patients.

In order to determine whether pneumococcus could be recovered with any regularity from dust the following study was carried out. The specimens of dust were collected as follows: A piece of paper was wrapped about a scrubbing brush which in turn was covered by a piece of cloth and then autoclaved. The dust was swept up with the sterile brush on to the paper which was then folded. This dust was mixed with sterile broth and 1 or 1.5 cc. of the mixture were immediately injected into the peritoneal cavity of a white mouse. Cultures were made from the heart's blood of the mouse.

In Table V are given the types of pneumococcus recovered from the dust of 62 rooms in which pneumonia had not occurred.

From these 62 specimens of dust, in 18 instances, or 29 per cent pneumococcus was recovered. In all but one instance these pneumococci belonged to those types which are normally found in the mouth. The specimen of dust from which the Type I pneumococcus was recovered came from a house where a known carrier of a Type I pneumococcus was visiting. From this study it is evident that pneumococcus can be easily recovered from dust. Furthermore, it appears that the strictly disease-producing types of pneumococcus, Types I and II, are not prevalent in dust when patients and healthy carriers of these types of pneumococcus are absent.

<sup>4</sup> Netter, L. D., *Compt. rend. Soc. biol.*, 1897, iv, series 10, 538.

<sup>5</sup> Emmerich, R., *Z. Hyg. u. Infektionskrankh.*, 1894, xvii, 167.

TABLE V.

*Incidence of Pneumococcus in Dust from Rooms in Which Pneumonia Had Not Occurred.*

Pneumococcus.	Incidence.	
		<i>per cent</i>
Type I.....	1	5.5
“ II.....	0	0
“ II a.....	0	0
“ II b.....	4	22
“ II x.....	3	16.6
“ III.....	2	11
“ IV.....	8	44.4
Pneumococcus present.....	18	
“ absent.....	44	
	62	

*Types of Pneumococci Obtained from Dust of Households in Which Pneumonia of Type I and Type II Had Occurred.*

Table VI shows the incidence and types of pneumococcus found in 183 specimens of dust from households where cases of Type I or Type II pneumonia had occurred.

TABLE VI.

*Incidence of Pneumococcus in Dust in the Presence of Pneumonia.*

Pneumococcus.	Incidence.	
		<i>per cent</i>
Type I.....	25	33.78
“ II.....	23	31.08
“ II a.....	0	0
“ II b.....	2	2.70
“ II x.....	2	2.70
“ III.....	2	2.70
“ IV.....	20	27.02
Pneumococcus present.....	74	
“ absent.....	109	
	183	

From 183 specimens of dust collected where cases of pneumonia due to pneumococcus of Type I or Type II had occurred, 74, or 40 per cent, showed pneumococcus. A Type I pneumococcus was found in 25 instances. In only one instance was a Type I pneumococcus recovered where a Type II pneumonia had occurred. This dust was collected 16 days after the patient had left his home. A Type II pneumococcus was recovered in 23 instances. In only one instance was a Type II pneumococcus found in the dust in the room where there had been a case of pneumonia due to Type I.

From this study it is evident that the highly parasitic pneumococci, Types I and II, are very prevalent in dust where cases of the same type of pneumonia have occurred. As the dust from only a comparatively small area of the floor was swept up, the organisms must have been present in a much larger proportion than these figures indicate.

In Table VII is presented a study of the types of pneumococcus obtained from the homes where a case of Type I or Type II pneumonia had occurred. As a rule, the dust was collected only from the patient's bedroom, but in some homes specimens were also obtained from the living-room and other bedrooms. Of 30 homes where a case of Type I pneumonia had occurred, in 13 instances, or 43 per cent, a Type I pneumococcus was recovered from at least 1 room. In 2 instances a Type I pneumococcus was recovered from 2 rooms. In all, 44 rooms were examined. From 15 of these rooms, or 34.08 per cent, a Type I pneumococcus was recovered. No Type II pneumococcus was recovered from a Type I home. Of 22 homes where a case of Type II pneumonia had occurred, in 13 instances, or 59 per cent, a Type II pneumococcus was discovered in the dust of at least 1 room. In 3 homes a Type II pneumococcus was recovered from 2 rooms. Of the 27 rooms which were examined, in 16, or 59 per cent, pneumococci of Type II were recovered. In only one instance was a Type I pneumococcus recovered from the dust of a Type II house. This occurred 16 days after the patient had left the house.

A comparison of Tables IV and VII shows that in the 28 Type I households in 3 instances the dust was positive, but no contact was found; in 2, the dust was negative, but a positive contact was present;

TABLE VII.

*Incidence of Pneumococci in Dust from Homes Where a Case of Pneumonia Occurred.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			days			
2,746	I	Patient's bedroom.	0	6	No pneumococci.	Dirty and dark.
2,804	I	" "	3	6	" "	
2,814	I	" "	3	2	" "	Clean, but dark.
		" "	11		" "	
2,815	I	" "	1	4	I	Light, moderately clean.
		" "	12		I	
2,816	I	" "	1	6	I	Dark, fairly clean, poorly ventilated.
		" "	10		No pneumococci.	
2,821	I	" "	1	6	I	
		" "	10		No pneumococci.	
2,824	I	" "	14	3	" "	Very dirty and dark.
2,825	II	" "	1	1	III	Small, dark.
		" "	13		II	
		" "	49		No pneumococci.	
2,827	II	" "	3	3	II	Clean, light, and small.
		" "	10		II	
		" "	19		No pneumococci.	
2,834	II	" "	5	5	IV	Moderately clean, fairly well ventilated.
		" "	16		I	
2,852	I	" "	2	4	No pneumococci.	Large, clean, and light.
		" "	23		" "	
2,853	II	" "	8	2	" "	Very clean, light, well ventilated.
		" "	23		" "	
2,854	II	" "	4	3	" "	
		" "	14		" "	
2,858	I	" "	2	2	I	Moderately clean and light.
		" "	14		IV	
2,868	II	" "	2	4	No pneumococci.	Moderately light, well ventilated.
2,869	II	" "	3	2	" "	Clean.
		" "	18		" "	
2,874	I	" "	3	3	" "	Clean.
		" "	10		" "	
2,879	II	" "	2	2	" "	
		" "	15		" "	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			<i>days</i>			
2,880	I	Patient's bedroom.	2	3	IV	Moderately clean,
		" "	13		IV	well ventilated.
2,881	II	" "	2	6	IV	Dark, poorly ven-
		2nd " "	2		IV	tilated.
		2nd " "	13		IV	
2,883	I	Patient's " "	5	4	No pneumococci.	Fairly clean but
		" "	15		" "	small.
2,885	II	" "	3	2	II	Clean.
		" "	13		No pneumococci.	
2,886	II	" "	3	4	II	Fairly clean but
		" "	14		No pneumococci.	dark.
2,890	II	" "	2	4	II	
		" "	15		No pneumococci.	
2,891	I	" "	1	3	" "	Clean.
		" "	23		" "	
2,892	II	" "	1	5	II	Moderately clean,
		" "	23		II	fairly well ven-
		" "	29		No pneumococci.	tilated.
2,896	II	" "	1	4?	II	Fairly clean, well
		" "	24		No pneumococci.	ventilated.
2,901	I	" "	2	5	I	Fairly clean, well
		" "	25		I	ventilated.
		" "	40		I	
		" "	50		I	
		" "	72		No pneumococci.	
		" "	92		" "	
2,906	I	" "	2	2	I	Large, clean.
		" "	21		No pneumococci.	
2,908	I	" "	3	5	I	Fairly clean.
		" "	18		No pneumococci.	
2,913	I	" "	2	5	" "	
		" "	13		" "	
2,917	I	Living-room.	8	6	II x	Fairly light.
		1st sick room.	8		No pneumococci.	Moderately clean.
		1st " "	22		" "	
		2nd " "	8		" "	

TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
2,922	II	Living-room.	3	3	II	Fairly clean, moderately ventilated.
		"	22		No pneumococci.	
		"	37		" "	
		"	70		" "	
		Patient's bedroom.	3		II	
		" "	18		II	
		" "	37		II	
		" "	57		II	
2,924	I	" "	70	5	No pneumococci.	Fairly light and ventilated.
		" "	3		" "	
		" "	21		" "	
		Living-room.	3		" "	
2,925	I	Patient's bedroom.	1	5	" "	Small, dark, moderately clean.
		" "	16		I	
		" "	29		I	
		" "	45		No pneumococci.	
		Living-room.	2		" "	
		" "	29		" "	
2,926	II	" "	45	4	" "	Clean.
		Patient's bedroom.	6		" "	
		" "	6		" "	
		" "	20		II	
		" "	39		II	
		" "	55		No pneumococci.	
2,934	II	" "	5	4	" "	Clean.
		" "	14		" "	
2,944	I	1st "	3	5	IV	House thoroughly cleaned before second dust specimens were collected.
		1st "	16		No pneumococci.	
		1st "	53		II x	
		2nd "	3		I	
		2nd "	16		No pneumococci.	
		Mother's room.	3		I	
		" "	16		No pneumococci.	
		" "	53		II x	
2,945	I	Living-room.	16	5	No pneumococci.	Dirty, dark, but fairly ventilated.
		"	3		I	
		"	17		IV	
		"	35		IV	



TABLE VII—*Continued.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of rooms.
			<i>days</i>			
2,945	I	Patient's bedroom.	3		IV	
		" "	17		IV	
		" "	35		IV	
2,946	II	" "	1	3	No pneumococci.	
		" "	17		" "	
2,949	I	" "	24	8	" "	
2,952	I	" "	3	2	" "	Fairly clean.
		" "	21		" "	Light, clean, well ventilated.
		Living-room.	3		" "	
		" "	21		" "	
2,954	I	" "	5	4	" "	
		" "	19		" "	
		Patient's bedroom.	5		" "	
		" "	19		" "	
2,955	I	" "	4	6	I	Moderately clean.
		" "	13		IV	
		" "	38		No pneumococci.	
		Living-room.	4		" "	
		" "	13		I	
		" "	38		No pneumococci.	
		" "	52		" "	
2,968	I	" "	3	5	IV	Fairly clean, fairly well ventilated.
		" "	19		No pneumococci.	
		Patient's bedroom.	3		" "	
		" "	19		" "	
2,971	II	" "	2	4	II	Well ventilated and clean.
		" "	26		No pneumococci.	
		Living-room.	2		II	
		" "	26		No pneumococci.	
2,976	II	" "	3	3	" "	
		" "	14		" "	
		" "	41		" "	
		Patient's bedroom.	3		" "	
		" "	14		II	
		" "	41		No pneumococci.	
2,984	I	" "	7	5	" "	Dark, dirty basement.
2,991	II	" "	4	1	II	

TABLE VII—*Concluded.*

Case No.	Type of infecting pneumococcus.	Room.	Time after removal of patient to hospital.	Day of disease on admission.	Type of pneumococcus.	Condition of room.
			<i>days</i>			
2,991	II	Patient's bedroom.	26		No pneumococci.	
		Living-room.	4		II	
		"	26		No pneumococci.	
2,996	I	"	1	3	II b	
		"	15		No pneumococci.	
		Patient's bedroom.	1		" "	
		" "	15		" "	
		Another "	1		I	
		" "	15		I	
M.M.	II	Patient's present room.	0	?	II	
		Patient's present room.	0		No pneumococci.	
F.I.	I	Patient's bedroom.	1	?	I	

*Summary.*

Type.	No. of pneumonia households examined.	Households giving rise to positive dust.		Total No. of rooms examined.	Positive rooms.	
			<i>per cent</i>			<i>per cent</i>
I.....	30	13	43	44	15	34.08
II.....	22	13	59	27	16	59.25
Total.....	52	26	50	71	31	43.66

in 8, the dust and at least one individual showed a Type I pneumococcus; while in 15, a Type I pneumococcus was recovered neither from the dust nor from a member of the household. In the 22 Type II households, in 10 instances the dust alone was positive; in 1, an individual alone was positive; in 3, a Type II pneumococcus was recovered from both the dust and from a member of the household; and in 8, neither the dust nor an individual showed a Type II pneumococcus. In these 50 households in 13 instances the dust alone.

was positive, while in only 3 was the dust negative in the presence of a positive contact.

Individuals or specimens of dust were studied from 54 households where a case of pneumonia due to a pneumococcus of Type I or Type II had occurred. Of the 30 Type I households, 14, or 46 per cent, showed either a positive human contact or a positive dust. From 24 Type II households 15, or 62 per cent, showed either a positive human contact or a positive dust. In other words, at the time of their admission to the hospital, the homes of over 53 per cent of the patients suffering from a pneumonia due to Type I or Type II were infected by a pneumococcus of the same type.

As a rule, the dust became negative before the contacts. But in two instances positive dusts were obtained after the carriers in the households had become negative. It is interesting that in the two homes which showed a positive dust for the longest time lived the two most persistent carriers. In Case 2,901, Type I, the dust was positive for 50 days, while the carrier had not become negative at the end of 80 days. In Case 2,922, Type II, the carrier was still positive at the end of 70 days and the dust was positive on the 57th day.

### *Two Pneumonia Epidemics.*

Through the courtesy of the State Department of Health and the local health officer of Rochester, N. Y., I have had an opportunity of studying two epidemics of pneumonia in institutions.

One epidemic occurred in a boys' asylum. At the time of the epidemic there were over 200 boys in this institution, but the 6 cases of pneumonia were limited to the boys occupying two of the four dormitories. 3 boys in each dormitory developed pneumonia; from the sputum of 3 of the 6 boys a Type I pneumococcus was recovered, from another a Type II x, and from a 5th a Type IV; from 1, no pneumococcus was obtained. The absence of Type I pneumococcus from 3 cases may be due to the fact that this study was not made until the patients were convalescent. The sputum of the other 56 boys who slept in the two dormitories was studied. From the saliva of 6, or 10 per cent, a Type I pneumococcus was isolated.

Three specimens of dust were taken at random from each of the

two dormitories. As these rooms were very clean this dust had to be swept out from between the chinks of the floor boards. One specimen of dust from each dormitory showed a Type I pneumococcus. Of the other four specimens two showed a Type IV, and from two no pneumococcus was obtained. Six specimens of dust from the vacant room used as a ward failed to show a Type I pneumococcus, but a pneumococcus of Type II and a pneumococcus of Type II b were recovered.

In this epidemic 50 per cent of the cases of pneumonia were found to be due to Type I pneumococcus; from 10 per cent of the healthy contacts and from the dust a Type I pneumococcus was recovered.

The other epidemic occurred in the Rochester State Hospital for the Insane. Here six cases of pneumonia occurred among the inmates of one ward of over 200 persons. A Type I pneumococcus was recovered from four of the patients, but the other two patients died before the type of infecting pneumococcus was determined. Each of the patients who died had shared a double room with one of the patients who showed a Type I pneumococcus. A Type I pneumococcus was also recovered from the dust of one of these two bedrooms. The saliva of 148 inmates of this ward was studied. In five instances a Type I, and in one a Type II pneumococcus were isolated. In all, nine specimens of dust were examined. In one instance a Type I, in one, a Type III, and in three, a Type IV pneumococcus was found.

In this epidemic six cases of pneumonia occurred in the ward of an institution. A Type I pneumococcus was recovered from four of the six patients, from 2 per cent of the healthy contacts, and from the dust.

#### *Related Cases of Pneumonia.*

Several instances which seem to be contact infections or infections from the same source have been studied. In the first instance a mother daily visited her son who was critically ill with a Type I pneumonia. The mother contracted a bad cold and developed pneumonia due to a Type I pneumococcus 11 days after her son was taken ill. Another case was that of an actor who was admitted to the hospital with a Type II pneumonia. The next day an actor who

shared the same dressing-room developed a Type II pneumonia. Before the end of the week an electrician at the same theater became ill with pneumonia. He also showed a Type II pneumococcus. In a third instance Miss H. (type of pneumonia not determined) was taken to a private hospital by Mrs. A. who developed a Type II pneumonia in a few days. Mr. B. helped to nurse Mrs. A. and shortly fell sick with a Type II pneumonia. In another instance a patient who had just recovered from a Type I pneumonia left the hospital March 9. The next day he went to see his brother who had developed a Type II pneumonia. On March 12 this patient, who had just recovered from a Type I infection, developed a pneumonia due to a Type II pneumococcus. The following case suggests the possibility of an infection by a healthy carrier. A patient was admitted to the hospital suffering from pneumonia due to *Pneumococcus* Type I. Specimens of sputum were obtained from the other members of the household. One 5 year old daughter was found to be a Type I pneumococcus carrier; the other two members of the household were negative. The dust from the room the patient had previously occupied in this house also showed a Type I pneumococcus. The little girl was sent to board with friends while her mother was in the hospital. She spent 3 days with the first family and then went to visit in the Bronx. 6 days after she left, a child in the home where she had visited came down with pneumonia due to *Pneumococcus* Type I. Specimens of sputum from the other members of this household were negative, but from the dust in the sick child's room a pneumococcus of Type I was isolated. The daughter of the original patient visited in the Bronx for 10 days. No cases of pneumonia developed in this home and the sputum of the members of this family as well as the dust failed to show the presence of *Pneumococcus* Type I. The child next went to visit friends in Brooklyn. Although the sputum from the members of this household were negative, from the dust a Type I pneumococcus was recovered.

#### DISCUSSION.

The results of the work detailed in this paper confirm the previous observations of Dochez and Avery on the occurrence of healthy carriers of disease-producing types of pneumococcus. Consideration

of the results of study over a period of years of the types of pneumococcus inducing lobar pneumonia shows that in the majority of instances infection is due to organisms belonging to Type I or Type II. The minority of cases, on the other hand, are due to infection with pneumococcus of Types III and IV.

Comparison of the types of pneumococcus obtained from the mouth secretions of normal persons with those isolated from individuals with lobar pneumonia shows the existence of two general classes of organisms. One of these, which consists of Types I and II, occurs only in association with disease. The other, which includes Types III and IV and the atypical Type II organisms, also causes pneumonia but these organisms are commonly found in normal healthy mouths. Rarely Types I and II have been found in the mouth secretions of normal individuals who give no history of association with cases of pneumonia. On the other hand, organisms of Types I and II have been found in 11 per cent of normal individuals who have been in intimate association with a case of pneumonia of the same type.

Although the presence of pneumococcus in dust has been known for some time, little significance has been attached to it. The results of this work show that pneumococcus can be easily recovered from dust. The types of pneumococcus found reflect accurately the pneumococcal flora of the mouth of the members of these households. Pneumococcus of Types I and II is rarely found in dust except where a case of pneumonia due to the same type of pneumococcus has occurred. In view of the ease with which dust can be disseminated it is not surprising that in a few instances a Type I or Type II organism was recovered from the dust which did not correspond to the type of pneumococcus producing the disease.

The occurrence of these disease-producing types of pneumococcus in the dust suggests the possibility that air-borne infection may play a part in the production of pneumonia. On the other hand, the mere presence of the disease-producing types of pneumococci in the mouth will not initiate disease. But if a susceptible individual comes in intimate contact with a case of pneumonia there is grave danger of his contracting the disease.

These facts suggest the following conclusions concerning the epidemiology of lobar pneumonia. Infection with pneumococcus of

Types I and II must be regarded as dependent upon either direct or indirect contact with a previous case of lobar pneumonia due to the same type of organism. These types of infection are either acquired by direct contact with a previous case of pneumonia, by association with a healthy carrier of one of these types of pneumococcus, or possibly by an air-borne infection from dust which has been infected. Infection with the sputum types of pneumococcus, namely Types III and IV and the atypical strains of Type II, may be autogenic, or due to the acquisition by the individual of one of these types to which he is especially susceptible.

#### SUMMARY.

1. Pneumococci of Type I and Type II are responsible for the majority of the cases of lobar pneumonia.

2. Among the pneumococci found in the mouths of healthy individuals Type IV predominates, Type III is frequent, and atypical organisms of Type II are occasionally found.

3. Healthy persons intimately associated with cases of lobar pneumonia may harbor in their mouth secretions the highly parasitic pneumococcus of Types I and II.

4. Occasionally a carrier of Type I or Type II pneumococcus is encountered in whom it is impossible to trace any contact with an infected patient.

5. From the dust of homes where cases of pneumonia due to Types I and II have occurred, pneumococci of the same type may be recovered.





## THE PRODUCTION OF ANTIPNEUMOCOCCIC SERUM.

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Ever since the demonstration in 1891 by Foa and Carbone,<sup>1</sup> Emmerich and Fowitzky,<sup>2</sup> and G. and F. Klemperer<sup>3</sup> of the immunizing properties of the serum of animals rendered immune to pneumococcus, this serum, modified in various ways, has been employed to a greater or less extent in the treatment of pneumonia in man.

Besides the employment of sera prepared in various ways in small series of cases, there has been an extensive employment of the sera prepared according to the directions of Pane,<sup>4</sup> Washbourn,<sup>5</sup> and more lately Römer<sup>6</sup> and Neufeld and Händel.<sup>7, 8</sup> In this country at least six commercial houses which sell biologic products have for some years been producing immune serum for the treatment of pneumonia and pneumococcus infections. It is impossible to know how extensively these sera have been employed, certainly to a sufficient extent to render their production profitable.

In spite of all that has been written concerning the theoretical principles involved in the preparation of antipneumococcic serum, and in spite of all the reports of its therapeutic application which have appeared, it is very difficult to learn from the literature on the subject exactly how these sera have been prepared or standardized.

<sup>1</sup> Foa, P., and Carbone, T., *Gazz. med. Torino*, 1891, xlii, 1.

<sup>2</sup> Emmerich, R., and Fowitzky, A., *Münch. med. Woch.*, 1891, xxxviii, 554.

<sup>3</sup> Klemperer, G., and Klemperer, F., *Berl. klin. Woch.*, 1891, xxviii, 833, 869.

<sup>4</sup> Pane, N., *Centr. Bakteriöl., 1te Abt.*, 1897, xxi, 664.

<sup>5</sup> Washbourn, J. W., *Brit. Med. J.*, 1897, i, 510.

<sup>6</sup> Römer, P., *Arch. Ophth.*, 1902, liv, 99; Experimentelle und klinische Grundlagen für die Serumtherapie der Pneumokokkeninfektion der menschlichen Cornea (Ulcus serpens), Wiesbaden, 1909.

<sup>7</sup> Neufeld, F., and Händel, *Z. Immunitätsforsch., Orig.*, 1909, iii, 159.

<sup>8</sup> Neufeld and Händel, *Arb. k. Gsndtsamte.*, 1910, xxxiv, 166, 1293.

Without this knowledge we can have no accurate starting-point from which to proceed toward improvements in methods of production.

In view of these facts and also since an antipneumococcic serum is now being prepared by The Rockefeller Institute for Medical Research and used in the Hospital of this institution, and since serum made in a similar manner is now being prepared by a number of commercial houses and by several public health laboratories for wider distribution, it has seemed important that an accurate description of the methods employed by us in its production be published, together with a brief discussion of the theoretical basis for the methods employed.

*Theoretical Considerations Concerning the Production of  
Antipneumococcic Serum.*

In spite of a general belief to the contrary, all kinds of animals, even the most susceptible, may be rendered actively immune to pneumococcus infection, by the previous injection of non-lethal doses of living pneumococci or even by the injection of the dead bodies. Indeed, the substances of the bacteria which give rise to the immune reaction are very resistant to various chemical and physical agents, and a review of the literature shows that it is possible to produce active immunity with a great variety of antigens prepared from the bacteria. The degree of immunity, however, differs, depending upon the procedures employed.

The serum of the actively immunized animals, in many cases, possesses protective and curative power, the degree of this power depending somewhat on the height of the active immunity, but not invariably or regularly so. It may be stated, however, that animals whose serum is protective and curative are always actively immune. On the other hand, an animal may itself be fairly highly immune without the serum containing any immune bodies that we can demonstrate, and without its having any demonstrable protective action. For instance, we have immunized rabbits so that they have successfully withstood 0.1 cc. of a living culture, of which 0.000001 cc. killed the control, without the blood showing a trace of immune bodies or any protective power. Quite frequently animals whose serum shows only moderate grades of immunizing value may be actively resistant

to enormous doses. The fact that active immunity and serum-immunizing power do not run exactly parallel does not necessarily indicate that active immunity in pneumococcus infection differs fundamentally from passive immunity, but suggests this possibility. At any rate, it suggests that in the reaction of resistance to, or recovery from, infection there may be other factors concerned than the humoral ones. Nevertheless, with present knowledge of serum therapy our effort must be confined to the production of a serum having a high content in demonstrable antibodies and a high protective value. The chief kinds of antibodies which can be demonstrated in this immune serum are agglutinins and opsonins or bacteriotropins, and the kind of protective power meant is that which is exhibited when a very small amount of the serum is injected into a susceptible animal such as the mouse, simultaneously with, or within a few hours following, the injection of a large dose of virulent culture, which alone would cause rapid death of the animal. These are the properties of the serum which are at present tested to indicate its therapeutic strength.

It would lead us far from our present purpose, were we to discuss here at length the mode of action of the serum, for the discussion would necessarily involve a consideration of the mechanism of resistance and recovery. It is not believed that the action of the immune serum is entirely dependent either on its power of causing agglutination (Bull<sup>9</sup>) or on its bacteriotropic power (Neufeld and Rimpau<sup>10</sup> and Neufeld and Händel<sup>7, 8</sup>), though these properties may play important parts. They, however, are susceptible of quantitative estimation. The protection of small animals undoubtedly reproduces more accurately the part which the serum plays in recovery from natural infection, as seen in the human patient, but even here the conditions are not identical.

At the present time, however, the effectiveness of the serum seems to be parallel to its content in the antibodies we have mentioned, and especially to its protective power tested as we have described. We believe the production of antipneumococcic serum that may be accurately standardized is of fundamental importance. Whether all theoretical qualifications are fulfilled is not so essential.

<sup>9</sup> Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

<sup>10</sup> Neufeld, F., and Rimpau, W., *Z. Hyg. u. Infectiouskrankh.*, 1905, li, 283.

*Specificity.*

The primary requisite for the serum is that it is specific. Everything stated above concerning both active and passive immunity is true only if the bacterium acted upon, either in antibody tests *in vitro*, or in protection tests *in vivo*, is identical with the organism used in producing the serum. This does not merely mean that the bacteria shall belong to the same species, for it is now generally known that different races of the same species of bacteria may show differences in their antigenic properties, even without differences in their cultural characteristics. These differences have heretofore, however, been considered differences merely in degree and mainly in lesser degree. To overcome the difficulties which this fact puts in the way of the production of immune serum for practical purposes, recourse has been had to the method of immunizing an animal with many different strains, producing a polyvalent serum. This method, for instance, has been made use of to a considerable extent in the production of antimeningococcic serum. Although certain races of meningococci differ from each other quite markedly in their immunological properties, yet they all have common characteristics which render a sharp differentiation difficult, if not impossible. The conditions as regards meningococci at present seem to be somewhat as follows. If we employ the Ehrlich nomenclature, each strain of meningococcus is endowed with a large number of kinds of receptors which we may designate by the letters of the alphabet. In one large group of strains of meningococcus *a* receptors predominate; in another group *b* receptors predominate, etc. In smaller groups other receptors, such as *k*, *l*, *m*, or *n* receptors, are most numerous. However, even in the first two groups, the *a* and *b* receptors are not exclusively present or even overwhelmingly predominating. Indeed each of the different races possesses practically all the different kinds of receptors but in greatly varying degrees.

Under these conditions, in order to produce a serum which will be active against all the different races it is necessary to choose and employ for immunization a large number of races in order that the entire receptor "spectrum" shall be covered as uniformly as possible. It seems that it is possible to do this fairly well as far as meningococcus is concerned.

The conditions as concerns pneumococcus are similar, though not identical. From agglutination experiments Kindborg<sup>11</sup> decided that all strains of pneumococci were immunologically distinct. Neufeld first brought evidence of group relationships and this fact has been elaborated and extended by the work in The Rockefeller Institute Hospital and elsewhere. Dochez and Gillespie<sup>12</sup> have demonstrated that the pneumococci obtained from cases of pneumonia occurring in this country belong in four large groups. The immunological characteristics of the organisms of three of these groups are very specific. The conditions therefore among pneumococcus are different from those obtaining among meningococcus. It is difficult to demonstrate by immunological methods that pneumococcus of Type I has any immunological characteristics (or receptors in the Ehrlich nomenclature) which are common to all pneumococci. Complement fixation tests, however, seem to show that the pneumococci of the different types do possess some common characters, at least these tests are not absolutely specific as regards the different types (Hanes<sup>13</sup>) and the antihemolytic reactions of specific sera against the hemolytic toxins derived from different types of pneumococci (Cole<sup>14</sup>) are not absolutely specific. So too the extremely active agglutinating immune sera produced by the injection of pneumococci of specific types may have slight degrees of effectiveness against certain strains of other types (Blake<sup>15</sup>). But in general the different types of pneumococci have a high degree of specificity as shown by protective action and by agglutination. Active immunity to the different types also seems very specific. This type specificity, which was only lately recognized, is of fundamental and primary importance in considering the practical application of immunity to therapy in this disease. Since nothing was known of this group specificity before the observations of Neufeld were made, it is very difficult to draw any conclusions from the observations or from the results of their practical application which were made in this field before that time. Where identical strains were

<sup>11</sup> Kindborg, A., *Z. Hyg. u. Infektionskrankh.*, 1905, li, 197.

<sup>12</sup> Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

<sup>13</sup> Hanes, F. M., *J. Exp. Med.*, 1914, xix, 38.

<sup>14</sup> Cole, R., *J. Exp. Med.*, 1914, xx, 346.

<sup>15</sup> Blake, F. G., *J. Exp. Med.*, 1917, xxvi, 67.

used throughout experiments, the conclusions of course were valid, but in the absence of knowledge of the importance of using only these strains, it is probable that in many cases this procedure was not strictly adhered to.

The work done in the Hospital of The Rockefeller Institute, both experimentally and clinically, indicates that immune serum against Type I infection is more effective than that against any other of the specific types. Indeed the results obtained both here and elsewhere indicate that this serum has great therapeutic value when it is employed in the treatment of cases due to the same type of pneumococcus. On the other hand, the observations which have so far been made with sera of the other types do not show that these produce distinct therapeutic effects. It is possible, of course, that improvements in the method of production or mode of application of these other types of serum may lead to more satisfactory results. For the present, however, we believe that the general practical application of this form of therapy should be confined to Type I cases, and this may readily be done since the type of infection in the individual case may easily be determined by the methods described elsewhere (Blake<sup>15</sup>). We also think it important at present that the commercial production of antipneumococcic serum for therapeutic purposes be confined to that effective against Type I infection. This paper aims to present the best method for manufacturing this serum and is based on the experience we have gained through the immunization of a very large number of rabbits, several goats, and four horses to Type I pneumococcus, four horses to Type II pneumococcus, one to Type III pneumococcus, and one to both Type I and Type II pneumococci, or ten horses in all.

#### *Animals to Be Employed for Immunization Purposes.*

For practical reasons small animals cannot be used to supply serum for therapeutic purposes, especially if the large amounts of serum which are now considered necessary are to be used.

In the earlier therapeutic tests, such as those of G. and F. Klemperer and Foa and Carbone, rabbits were used for obtaining the small amounts of serum used. Later Washbourn used ponies for immunizing purposes. Pane used a cow and an ass. Neufeld used horses in the preparation of his serum.

Römer<sup>6</sup> has laid stress on the importance of combining the sera prepared by immunizing a number of different animals. His reason for this is a purely theoretical one; namely, that all the different antigenic elements contained in a complex structure such as the pneumococcus may not find suitable receptors in any one animal. With present knowledge it seems that we may disregard this consideration.

We believe that for practical reasons it is best to use horses. Our experience is not sufficient to enable us to have a definite opinion as to the kind of horses best to choose for this purpose; apparently, however, draft horses are superior to the lighter grades and to the more finely bred animals. Joint injuries, indeed injuries of all kinds, undoubtedly contribute to the localization of pneumococcus and consequent focal infection. The highly strung, nervous horses are more liable to these injuries and infections. These accidents delay immunization. As regards antibody response, there has been marked individual variation, but the difference has not been associated with any obvious distinction in type of horse. In rabbits, with which our experience is much larger, there is very great individual difference. It is far more important in the production of this kind of serum than in the production of antitoxic sera that the horses should be perfectly sound and healthy, especially without old joint injuries.

#### *Site of Injection.*

Neufeld has laid stress on the value of intravenous injections. We have also employed this method since local reactions are thereby avoided. With dead cultures the local reactions are usually not severe or important; where living cultures are employed, however, they may be violent and abscess formation not infrequently occurs. It is felt, moreover, that by intravenous injections the bacteria are brought more rapidly and quickly and with less opportunity for change into contact with widely distributed cells, the reaction with which is supposed to result in the immunity response.

#### *Choice of Antigen.*

*Use of Living or Dead Cultures.*—As we have previously stated, the production of active immunity is attended with little difficulty and the form of antigen, so far as we know, is not of great importance,

but when we come to the question of the production of humoral immunity, especially of the highest grade, this factor may be of the greatest importance. It has generally been assumed on theoretical grounds that to produce the highest grade of humoral immunity it is necessary to use living cultures. It seems likely that all the various constituents of the bacterial cell and even some products of growth, which may be very labile, give rise to specific antibodies. Therefore to obtain the most varied and complete reaction it has been thought necessary to employ the living organisms, which may, for a short time at least, grow in the body and produce or give rise by their dissolution to certain labile metabolic substances which may stimulate antibody formation. Most of the antipneumococcic sera which have been prepared, at least of late years, have been produced by the injection first of dead, then of living organisms. The general acceptance of this method is probably due in part to what is known concerning immunity in tuberculosis. Here, as is well known, the only immunity that is effective has been produced by the injection of living organisms, by producing a mild tuberculous infection. One must be careful, however, in applying what is known concerning one type of infection directly to another, without due consideration. As knowledge of infection increases it becomes more and more evident that each disease must be considered independently. As we shall show later, it is possible that in the production of antipneumococcic serum the living organisms are not so important as they have previously been considered.

*Virulence of Organisms Employed.*—Pneumococci may rapidly lose their virulence for animals when grown in an artificial medium. This loss of virulence, however, may not be uniform for all kinds of experimental animals. Moreover, the virulence of pneumococci which have long been grown on artificial media may be increased by passing repeatedly through experimental animals. Here again the increase of virulence may not be uniform for all the varieties of animals. It is possible, for instance, to obtain a given strain which is very virulent for guinea pigs, with little virulence for rabbits. Even for animals as closely related in their susceptibility to pneumococcus infection as the rabbit and mouse, it is possible to have races which are highly virulent for one with little virulence for the other.



It is manifest, therefore, that we can judge only very imperfectly of the virulence of a given race for man by determining its virulence for a susceptible animal, such as the mouse or rabbit. Whether in the production of an immune serum it is important for the organisms employed to be highly virulent or not is not known. Neufeld and his associates<sup>7, 10</sup> have laid stress on the employment of virulent cultures, believing that the action of immune serum is to neutralize exactly those receptors of the bacterium upon which virulence depends. They state that they have proved experimentally that immunity cannot be produced with avirulent races, but give no protocols. Races virulent for mice have been employed in all our immunizing studies and we have no observations concerning immunization with avirulent races. If virulence of the organism used as antigen is important, what we have just stated concerning virulence for different species of animals becomes of great significance. It will be remembered that mice are used in all our tests of immunity. The fact that a serum is protective for mice would indicate that it had been produced by a race of pneumococci virulent for mice, but the test might give us little information relative to its protective power for man. We have therefore come to believe, on these purely theoretical grounds, that the immunization of horses should be carried on with organisms which have not been long under artificial cultivation since their isolation from the human body. These organisms are practically always virulent for mice, so that where they are employed, the test of the protective power of the serum in mice is probably a good test for protective and curative power in man. In order to have a culture which has not long been removed from the human body always ready for use, advantage may be taken of an observation made by Heim<sup>16</sup> and confirmed by Neufeld and Händel<sup>7</sup> and abundantly corroborated by us; namely, that when pneumococci are preserved in pieces of dried tissue or blood they remain viable for a very long time, and also retain their virulence undiminished. For preserving small amounts of culture the spleens of infected mice, dried and kept in a vacuum, are most satisfactory. For preserving large amounts of culture from human patients, it is well to inoculate a rabbit with blood or other infected material. After infection has reached a maximum grade,

<sup>16</sup> Heim, L., *Z. Hyg. u. Infektionskrankh.*, 1905, 1, 123.

the rabbit is bled and the blood is spread in thin layers in Petri dishes and dried. To obtain a fresh culture at any time all that is necessary is to inoculate a little of this dried material into the peritoneal cavity of a mouse and later make a fresh culture from the heart's blood. For immunizing purposes a fresh culture obtained in this way should be prepared every 1 or 2 weeks. This precaution in obtaining cultures should be taken not only when the live bacteria are to be injected, but also when dead organisms are to be employed.

*Method of Growing Organisms Used for Injection.*—The use of bacteria grown in broth and separated by centrifugalization from the medium in which they are grown was first employed by Neufeld<sup>17</sup> who believed that the substances formed in the medium during growth are not useful but indeed harmful. We also think that it is not necessary to use the fluid in which the bacteria are cultivated. In this belief we differ from Wadsworth<sup>18</sup> whose observations apparently show that the serum produced with whole cultures is more effective than that produced by the injection of the bacteria alone. His experiments, however, are not entirely conclusive and in the absence of any method of accurately titrating this increased efficacy, it does not seem advisable to employ the whole culture. Moreover, the injection of the whole culture adds greatly to the difficulty of the immunizing process. As is well known, even fresh bouillon is toxic and after bacterial growth has taken place it is still more toxic. In using whole cultures, therefore, one is much restricted as to the amount that can safely be injected. When the amount of culture to be injected reaches a large size, the technical difficulties and time required in centrifugalizing the cultures become considerable. We have attempted to overcome these difficulties by growing the pneumococci on blood glucose agar in flasks and washing off the surface growth in salt solution, using the emulsion so obtained, without centrifugalizing. For certain theoretical reasons, moreover, we thought this method might be of advantage. In our experience, however, the method of growing in bouillon and centrifugalizing still proves the most satisfactory. In our earlier work the organisms were obtained by growing in broth,

<sup>17</sup> Neufeld, F., *Z. Hyg. u. Infektionskrankh.*, 1902, xl, 54.

<sup>18</sup> Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.

centrifugalizing, and washing once in salt solution, and then re-suspending in salt solution. In our later work, however, we have not thought it necessary to wash, but have merely centrifugalized and then made an emulsion of the sediment in salt solution.

For obtaining a satisfactory growth in bouillon the reaction of the medium is of great importance. Pneumococci grow best in a medium the reaction of which is 0.3 to 0.5 per cent acid to phenolphthalein. If the reaction is more acid than this, a satisfactory growth may not be obtained. We have added no sugar, serum, or other enriching substance to the medium employed.

#### *Size and Spacing of Doses.*

In our first studies we employed the method which has been largely employed by others, making the injections every 7 to 8 days. This is the method generally employed in immunization and has developed from the observation that the most efficient stimulus can be applied at the time when the immunological response to the preceding dose is most active. Grades of high immunity have been thought to increase in a step-like manner, each increment being added to that previously present. It is again not certain, however, that anti-pneumococcus immunity obeys the same laws as other forms of immunity in which this step-like rise occurs. Certain of our observations to be mentioned later indicate that even when the injections are made at the period of greatest activity, instead of a rise in the immunity, there may be a fall, especially if the dosage is too large. After a primary immunity had been obtained by weekly injections of dead cultures, live organisms were injected, beginning with small doses, *i.e.*, the bacteria from 2 to 5 cc. of bouillon culture, and the succeeding weekly injections were gradually increased in size up to the bacteria contained in 1 or even 2 liters of culture. Neufeld speaks of injecting doses of living pneumococci as large as the bacteria obtained from 1,500 cc. of culture in the horse, and from 3,500 cc. in the ass. He injected doses of dead organisms as large as the bacteria contained in 9 liters of culture. In our experience this method of immunization is attended with many disadvantages. It has required 6 to 8 months to bring horses up to the desired grade of immunity. That these very large doses are not necessary is shown

by the following protocol of a horse in which the attempt was made to produce an effective serum by using only small doses of culture (Table I).

TABLE I.  
*Horse 1. Immunized to Pneumococcus Type I.*

Date.	Injection.	Tests of serum.
1916		
Feb. 2	3,250 units of tetanus antitoxin subcutaneously.	
" 4	Bacteria from 25 cc. of culture, killed by heating.	
" 11	" " 50 " " " " "	
" 18	" " 75 " " " " "	
" 26	" " 100 " " " " "	
Mar. 3	living, from 2.5 cc. of culture.	
" 11	" " " 5.0 " " "	
" 19	" " " 10.0 " " "	
" 29	" " " 20.0 " " "	
Apr. 3	" " " 40.0 " " "	
" 15	" " " 80.0 " " "	
" 22		Agglutination:* complete. Protection:† 0.1 cc., D.; 0.01 cc. S.
" 22	Bacteria, living, from 120 cc. of culture.	
May 7		Agglutination:* complete. Protection:† 0.1 cc., S.

\* These tests were made before we commenced the routine accurate titration of the agglutination strength.

† In the protection tests each of the mice received 0.2 cc. of serum simultaneously with a graduated dose of culture, both given intraperitoneally. The figures given indicate the amount of culture added. D. indicates died; S., survived. For brevity, only the highest dose with which recovery took place is given. In all cases the control animals receiving 0.000001 cc. of culture alone died.

It is true that the results obtained with this horse were unusually good, better than any we have since been able to obtain with a similar method. They indicate, however, that the large doses which we had been using were not necessary, that equally good results could be obtained with much smaller amounts of culture.

*Modification and Improvements in the Methods of Immunization.*

Over a year ago we undertook experiments to determine whether or not animals could be immunized more rapidly than had been done in the past, and also whether it might not be possible to obtain a higher grade of immunity than we had previously observed, and finally to determine for ourselves whether the use of living organisms is necessary in producing humoral immunity.

To determine the best methods of immunization a large number of rabbits was immunized in various ways and the development of immunity studied. Certain observations which had been made indicated that the process might be hastened by more frequent injections of antigen than had previously been used.

In 1900 Dean<sup>19</sup> showed that in the production of diphtheria antitoxic immunity the administration of the toxin at 3 day intervals gave very successful and practical results. Daily doses of antitoxin have been administered with good results when other methods have failed.<sup>20</sup> In 1908 Fornet and Müller<sup>21</sup> showed that precipitating sera could be produced very rapidly by three daily injections of antigen, bleeding on the 12th day. Bonhoff and Tsuzuki<sup>22</sup> confirmed these observations and Tsuzuki<sup>23</sup> showed that by a similar method a rapid production of typhoid-agglutinating serum could be produced. Similar observations have been made by Gay and his assistants.<sup>24</sup>

Flexner and Amoss<sup>25</sup> have employed a similar method in the production of antidyenteric serum, injecting live cultures on 3 successive days, with excellent results. In the same way the method of three daily injections has been employed by Amoss and Wollstein<sup>26</sup> in the production of antimeningococcic serum.

In the production of antidyenteric and antimeningococcic serum stress has been laid on regulating the size of the dose so that, following each inoculation, a febrile reaction shall be obtained. It occurred to us that the choice of three daily doses had been made more or

<sup>19</sup> Dean, G., *Tr. Path. Soc. London*, 1900, li, 15.

<sup>20</sup> Personal communication from Dr. Theobald Smith.

<sup>21</sup> Fornet, W., and Müller, M., *Z. biol. Techn. u. Method.*, 1908-09, i, 201.

<sup>22</sup> Bonhoff, H., and Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 180.

<sup>23</sup> Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 194.

<sup>24</sup> Gay, F. P., *Ergebn. Immunitätsforsch. exp. Therap., Bakteriolog. u. Hyg.*, 1914, i, 231.

<sup>25</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

<sup>26</sup> Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

less arbitrarily and that possibly daily doses administered over a longer time might be still more efficacious in producing a satisfactory result, especially since this method had proved of value in the production of diphtheria antitoxin. That this method is indeed of much value is seen from the results of the experiments given below. The experiments need not be described here in detail. Certain slight variations due to external causes were made in individual instances, but most of the animals were treated about as follows.

*Series 1.*—These animals received weekly intravenous injections of large amounts of bacteria obtained by centrifugalization of broth cultures, which were then killed by heating for 1 hour at 56°C. The size of the doses varied from the bacteria contained in 100 cc. of broth to those contained in 500 cc.

*Series 2.*—These animals received intravenous injections of very small amounts of bacteria killed by heat as in the above experiment; the doses were given daily for 7 days, then an interval of 7 days was allowed to elapse, and a second series of injections was given, etc. The bacteria in the individual doses varied from those contained in 1 cc. of broth to those contained in 2 cc.

*Series 3.*—These animals received combined doses of immune horse serum and living cultures intravenously, beginning with 1 cc. of serum plus 0.1 to 0.5 cc. of culture. This dose was repeated every day for 3 days, then 7 days were allowed to elapse, and a second series of these combined doses was given, this time using slightly larger doses of culture. After an interval of another week, combined doses using still larger amounts of culture were given, etc.

*Series 4.*—These animals received varying sized doses of pneumococci killed by the addition of 0.5 per cent carbolic acid. The bacteria were centrifugalized from broth cultures, taken up in salt solution, the carbolic acid was added, and the mixture kept at 37°C. over night. As in the preceding series, the injections were given intravenously on 3 successive days, then an interval of a week was allowed to elapse, and the second series given. The number of bacteria in the individual doses varied from those contained in 2 cc. of broth to those in 50 cc. of broth. Some animals received large weekly doses subcutaneously.

*Series 5.*—These animals received injections of an antigen prepared as follows. The bacteria were grown in broth, centrifugalized, washed, and taken up in a very small amount of salt solution, and this emulsion was added to a large amount of acetone. The sediment which formed was centrifugalized at once, dried in a vacuum, and after 12 hours in the vacuum, taken up in salt solution and thoroughly shaken. From this somewhat viscid translucent fluid further dilutions in salt solution were made. The injections in these rabbits were made once a week, and the doses varied from an amount of antigen representing the bacteria contained in 5 cc. of broth to one representing the bacteria contained in 220 cc. of broth. The small doses were given intravenously, the larger ones subcutaneously.

In addition to the above, a small series of rabbits received injections of antigen prepared by freezing and grinding the bacteria, and another small series received injections of bacteria dissolved in bile.

In studies such as these it must be borne in mind that individual rabbits immunized in exactly the same way may show quite marked variations in their immunity response. Slight differences, therefore, in results obtained in small series of animals are not of significance.

It would lead us too far to attempt to analyze in detail the results in the various tests or to publish the protocols. One definite fact stands out from these studies. Uniformly the results following the injection of small doses of killed culture given daily over a period of 7 days followed by 7 days of rest were excellent (Series 2). This is in marked contrast to the results obtained by the injection of large doses of killed cultures given at intervals of a week (Series 1). Serum from these animals showed little or no evidence of immunity. This is strikingly shown in Table II.

TABLE II.

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
Rabbit 1.					
1916		gm.			
May 27	1	1,670	Bacteria from 250 cc. of culture, killed by heating.		
	7 day interval.				
June 4	9	1,400	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 14	19	1,300	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 24	29	1,270	Bacteria from 250 cc. of culture, killed by heating.		
	6 day interval.				
July 1	36	1,150		Undiluted, 0	0.001 cc., D.

TABLE II—Continued.

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
Rabbit 2.					
1916 May 28	1	gm. 1,520	Bacteria from 1 cc. of culture, killed by heating.		
" 29	2		Bacteria from 1 cc. of culture, killed by heating.		
" 30	3		Bacteria from 1 cc. of culture, killed by heating.		
" 31	4		Bacteria from 1 cc. of culture, killed by heating.		
June 1	5		Bacteria from 1 cc. of culture, killed by heating.		
" 2	6		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 10	14	1,650	Bacteria from 1 cc. of culture, killed by heating.		
" 11	15		Bacteria from 1 cc. of culture, killed by heating.		
" 12	16		Bacteria from 1 cc. of culture, killed by heating.		
" 13	17		Bacteria from 1 cc. of culture, killed by heating.		
" 14	18		Bacteria from 1 cc. of culture, killed by heating.		
" 15	19		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 23	27	1,180	Bacteria from 1 cc. of culture, killed by heating.		
" 24	28		Bacteria from 1 cc. of culture, killed by heating.		
" 25	29		Bacteria from 1 cc. of culture, killed by heating.		
" 26	30		Bacteria from 1 cc. of culture, killed by heating.		
" 27	31		Bacteria from 1 cc. of culture, killed by heating.		
	8 day interval.				
July 6	40	1,100		1: 200 + 1: 400 0	0.2 cc., D. 0.1 cc., S.



As the protocols show the serum of Rabbit 1 injected weekly with large doses of culture showed no agglutinating power and only moderate protective action. On the other hand, the serum of Rabbit 2 which was treated over practically the same period of time but received very small daily doses showed high agglutinating strength, positive in a dilution of 1:200, and high protective power. These results were most surprising and striking, especially as all of the eight rabbits inoculated with the small daily doses showed an extraordinarily prompt and active response, while all of the eight rabbits inoculated with the large doses showed very little or no response in the same period of time.

The studies of other methods of immunization gave little information. The attempts to produce immunity with combined doses of serum and culture (Series 3) gave unsatisfactory results. The experiments of this series were undertaken because by this method live cultures could be injected at the very beginning of the immunization. Moreover, Theobald Smith<sup>27</sup> and von Behring<sup>28</sup> have shown the possibility of immunizing with combined doses of toxin and antitoxin. Besredka<sup>29</sup> and others have shown the possibility of immunizing against typhoid with sensitized cultures. Levy and Aoki<sup>30</sup> claim to have produced immunity to pneumococci with great rapidity (in 6 hours) by the injection of sensitized bacteria killed with carbolic acid. However, in view of what is now known concerning possible dissociation of pneumococcus antigen and antibody (Gay and Chickering<sup>31</sup>), it is possible that what the latter writers observed was not active immunity but slight grades of passive immunity. In any case, the grade of immunity produced was slight. Our experiments yielded no evidence in favor of the combined injection of culture and immune serum. Nevertheless, it is possible that other modifications of the method might yield better results, especially as we made no effort to balance accurately the amounts of culture and immune serum employed.

<sup>27</sup> Smith, Theobald, *J. Med. Research*, 1907, xvi, 359.

<sup>28</sup> von Behring, E., *Deutsch. med. Woch.*, 1913, xxxix, 873.

<sup>29</sup> Besredka, cited by Gay.<sup>24</sup>

<sup>30</sup> Levy, E., and Aoki, K., *Z. Immunitätsforsch., Orig.*, 1910, vii, 435.

<sup>31</sup> Gay, F. P., and Chickering, H. T., *J. Exp. Med.*, 1915, xxi, 389.

The serum of the rabbits of Series 4 inoculated with cultures killed by carbolic acid indicated no considerable grade of immunity. There is apparently no advantage to be gained in employing antigen prepared in this way. Levy and Aoki,<sup>30</sup> however, have reported the production of immune serum in dogs by the use of this method; but the grade of immunity, according to our standards, was slight.

Finally, the experiments in Series 5 and those in which frozen and ground bacteria and bacteria dissolved in bile were employed simply offer observations in the use of bacterial antigenic substances produced in other ways.

Neufeld<sup>32</sup> and Vetrano<sup>33</sup> have also employed bile extracts of pneumococci for immunizing purposes, only, however, in the production of active immunity. Others have employed still other artificial methods of treating the bacteria. G. and F. Klemperer<sup>3</sup> used glycerol extracts and Wadsworth<sup>18</sup> attempted immunization with cultures precipitated with alcohol and dissolved in water.

These and our own observations with artificially produced antigens show that slight grades of immunity can be produced by antigens prepared by various methods. The antigenic substance seems to be highly resistant. However, neither the observations of others nor our own indicate that these methods are especially useful in extracting the antigenic substance or in rendering it more effective. Indeed in all instances the immunity reaction resulting from the employment of antigens prepared in these ways was less intense than that following the injection of heat-killed bacteria. One fact emerges from these experiments, however, though in a less striking way than from the observations previously mentioned; namely, that small doses repeated frequently are much more effective than large doses given at longer intervals. The very large doses seem to have a definite repressing action on the development of antibodies.

These observations led us to immunize a series of four rabbits with small daily doses to determine the exact time of appearance of the immune properties in the serum. Table III is a typical protocol of one of the rabbits.

<sup>32</sup> Neufeld, Z. *Hyg. u. Infektionskrankh.*, 1900, xxxiv, 454.

<sup>33</sup> Vetrano, G., *Centr. Bakteriok., 1te Abt., Orig.*, 1909, lii, 275.

TABLE III.

*Rabbit 3.*

Date.	Weight.	Injection of vaccine.	Agglutination tests.	Protection tests.
1916	gm.	cc.		
May 29	1,950		0	0.0001 cc., D. 36 hrs. 0.00001 " " 36 " 0.000001 " " 36 "
" 29	1,950	1*		
" 30		1		
" 31	1,900	1		
June 1		1		
" 2		1		
" 3		1		
" 9	1,910		1:1 $\frac{1}{2}$ ++ 1:10 0	0.1 cc., D. 16 hrs. 0.01 " " 79 " 0.001 " S. 0.0001 " "
" 11		1		
" 12		1		
" 13	1,900	1		
" 14		1		
" 15	1,750	1		
" 16		1		
" 23			1:1 ++ 1:10 ++ 1:20 +	0.1 cc., S. 0.01 " " 0.001 " "
" 24		1		
" 25	1,750	1		
" 26		1		
" 27	1,900	1		
" 28		1		
" 29	1,850	1		
July 5	1,950			
" 7			1:100 ++ 1:200 + 1:400 0	0.2 cc., D. 20 hrs. 0.1 " S. 0.01 " "

\* In these experiments the material for injection was prepared at the beginning of the experiment and kept on ice. 150 cc. of an 18 hour broth culture of *Pneumococcus* Type I was centrifugalized and the sediment washed once in salt solution. The sediment was taken up in 10 cc. of salt solution and heated  $\frac{3}{4}$  hour at 56°C. Cultures were sterile. The emulsion was kept on ice and, after shaking, a small amount was removed and diluted to original volume before each injection. The injections were made intravenously.

The experiment shows that it is possible by this method of immunization to produce a high grade of immunity within 6 weeks; even within 4 weeks a considerable grade of immunity is present. The serum from each of the four rabbits showed a high agglutinating titer and a constant protective power against 0.01 to 0.1 cc. of culture. Two of the rabbits lost slightly in weight, one remained stationary, and one actually gained. They showed no other ill effects.

Experiments have also been made to show whether similar or better results could be obtained by injecting animals daily with small doses over longer periods than 7 days. Four rabbits were given small daily doses for 14 days and then bled and the serum was tested on the 5th and 10th days following the last injection. The serum of these

TABLE IV.  
*Horse 2. Immunized to Pneumococcus Type I.*

Date.	Injection.	Agglutination test.	Protection tests.
<i>1916</i>			
Jan. 27	1,500 units of tetanus antitoxin subcutaneously.		
" 29	Bacteria from 50 cc. of culture, killed by heating.		
" 30	" " 50 " " " " " "		
" 31	" " 50 " " " " " "		
Feb. 1	" " 50 " " " " " "		
" 2	" " 50 " " " " " "		
" 3	" " 50 " " " " " "		
" 10	" " 50 " " " " " "		
" 11	" " 50 " " " " " "		
" 12	" " 50 " " " " " "		
" 13	" " 50 " " " " " "		
" 14	" " 50 " " " " " "		
" 15	" " 50 " " " " " "		
" 23	" " 50 " " " " " "		
" 24	" " 50 " " " " " "		
" 25	" " 50 " " " " " "		
" 26	" " 50 " " " " " "		
" 27	" " 50 " " " " " "		
" 28	" " 50 " " " " " "		
Mar. 8		1: 200 +	0.1 cc., S. 0.1 " D. 0.01 " S.

animals has shown very slight agglutinating and protective power, so that this modification seems to be of no advantage.

The results of the rabbit experiments have led us to try the method of immunization described above in horses. The result obtained in one horse treated in this manner is given in Table IV.

The results in this and other horses have shown that it is possible in this way to produce a very high grade of primary immunity with a great saving in time and without danger to the animal. The dosage has been arbitrarily chosen. It is possible that with further experience and more carefully regulated dosage it may be possible to produce even higher grades of immunity in this way. It may even be possible by this method to obviate entirely the use of live cultures.

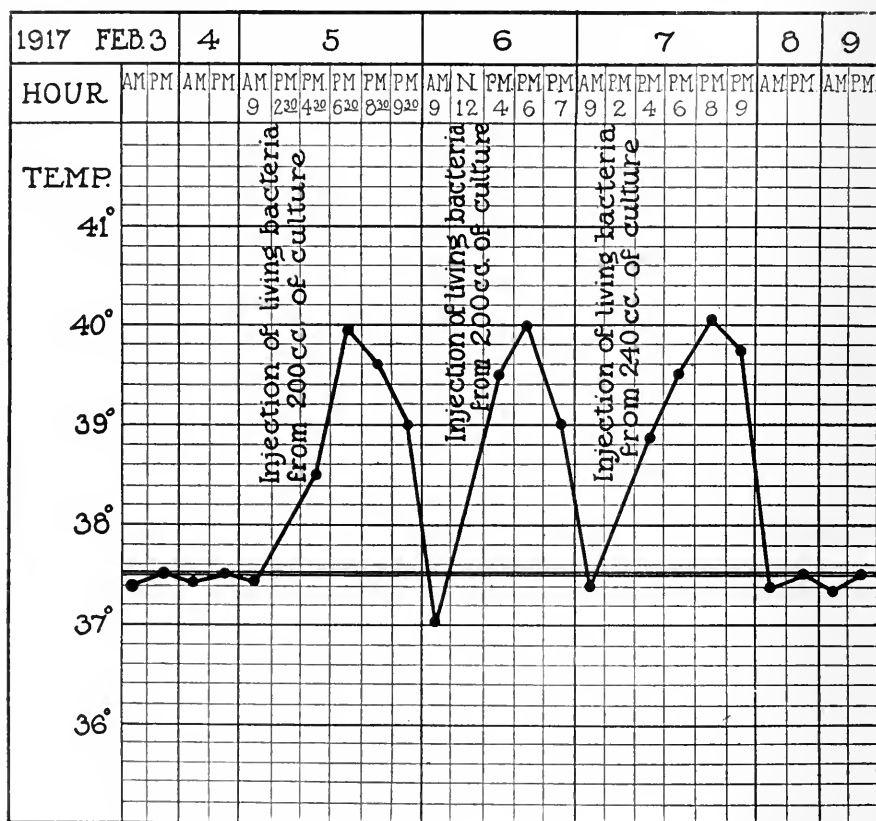
What has been attempted so far, however, has been the production of a primary immunity in the quickest possible time and with the least loss in horses.

TABLE V.

*Horse 2, Undergoing Immunization Since January 29, 1916. Last Bleeding, 11 Liters, on May 15, 1917.*

Date.	Injection.	Protection tests.
1917		
May 26	Bacteria from 100 cc. of culture, killed by heating.	
" 27	" " 100 " " " " " "	
" 28	" " 100 " " " " " "	
" 29	" " 100 " " " " " "	
" 30	" " 100 " " " " " "	
June 1	" " 100 " " " " " "	
" 9	" " 100 " " " " " "	
" 11	" " 100 " " " " " "	
" 14	" " 100 " " " " " "	
" 15	" " 100 " " " " " "	
" 16	" " 100 " " " " " "	
" 17	" " 100 " " " " " "	
June 25	Blood test.	0.01 cc., D.
July 6	Bacteria, living, from 100 cc. of culture.	
" 7	" " " 130 " " "	
" 8	" " " 160 " " "	
" 13	Blood test.	0.2 cc., S.

The problem now confronting us is to determine the method by which horses having an established primary immunity may be brought up to the highest possible level and kept there. In order to do this, when dead cultures have failed, it may be necessary to resort to living



TEXT-FIG. 1. Temperature curve of Horse 3 injected with living *Pneumococcus* Type I.

cultures. An observation on one of our horses makes this probable (Table V).

In this horse, whereas repeated small daily doses of killed culture failed to bring the serum up to full strength, three moderate sized doses of living culture caused the serum to acquire maximum power.

This result has been seen on numerous occasions and it is probable that in most horses living cultures must finally be employed. When injections of living cultures are made we now employ the method described by Flexner and Amoss,<sup>25</sup> administering three doses in amounts sufficient to produce a moderate febrile reaction. A typical curve is shown in Text-fig. 1. So far as pneumococcus immunity, however, is concerned, the necessity for producing febrile reactions is not established. Neufeld believed that so far as agglutinating sera are concerned the power depends not so much on the height of the immunity as on the intensity of the last reaction through which the animal had passed. Therefore he thought it advisable to inject as large amounts as possible without killing the animal. The observations we have made do not support this point of view. In our experience with horses a violent reaction is not always followed by a marked immunity response or increase in agglutinating power of the serum; indeed the opposite is frequently the case. For the present, however, in giving live cultures it is probably better to be guided in the size of the dose by the febrile reactions. We think, however, that large doses should be avoided even though the febrile reaction is slight.

*Typical Method of Immunization Based on Previous Observations.*

As a result of our observations and those of others we now believe that immunization should be carried out according to the method as at present employed by us, which is briefly given below.

Having obtained a sound, fairly heavy horse, it is first given a glanders test. At present the complement fixation test is used for this purpose. A specimen of 20 cc. or more of blood is obtained before any treatment is given and is kept for use in control tests. The immunization is then carried out as follows. All injections are made intravenously, employing for this purpose a Luer syringe. To avoid any accidental injury to the vein it is well to have the needle attached to the syringe with a small piece of rubber tubing. The culture used for injection, whether living or dead, should be one highly virulent for mice, 0.000001 cc. killing regularly, and it should have gone through very few passages in animals or on artificial media since removal from the human patient. The method for keeping the cultures is described above (page 545). In preparing the material for injection, both living and dead, the organisms are grown on beef peptone broth, reaction 0.3 to 0.5 per cent acid to phenolphthalein. Cultures about 12

to 15 hours old are preferable as at this time maximum growth is present, with a minimum of autolysis (Chesney<sup>34</sup>). The cultures should contain about 200 to 300 million bacteria per cc. The culture is centrifugalized until the supernatant fluid is clear. With the large centrifuge employed by us this requires about 20 to 30 minutes. The supernatant fluid is then poured off and the sediment is taken up in a small amount of sterile salt solution.

If the organisms are to be injected alive, the emulsion in salt solution is not made until just before injection, so that autolysis and death may not take place. If the organisms are to be injected dead, the emulsion is placed in a tube in a water bath and kept at 56°C. for  $\frac{3}{4}$  hour. For the daily injections a considerable amount of the emulsion, after killing, is prepared and kept on ice. This may be employed for all the injections in the series of 6 to 7 days. We think, however, a fresh emulsion should be prepared each week. For each injection the dilution of the fluid should be such that the volume injected is about 20 cc.

The following course of injections is now carried out. Every day for 6 days an amount of the emulsion of killed pneumococci containing the bacteria from 50 cc. of the bouillon culture is injected. An interval of 7 days is allowed to elapse and then a second series of daily injections, of the same size, is made. Again an interval is allowed to elapse and on the 6th day a specimen of blood is obtained for testing. Tests are made at once for agglutinating and protective power. This requires several days.

If the serum causes agglutination in a dilution of 1:200 and is of standard protective value, 0.2 cc. regularly protecting a mouse against 0.1 cc. of a virulent culture, bleeding may be carried out at once; that is, on the 10th to 12th day following the last injection. As a matter of fact, we have never seen the titer of the serum after this amount of treatment to be so high. Consequently it has been our practice, and we advise, that 8 to 10 days after the last injection of the second series of dead bacteria, injection of live organisms be commenced. These injections are given on successive days. The first injection should consist of the bacteria contained in 20 cc. of the original culture. The temperature is taken every 2 hours for 8 to 10 hours following each of the injections of live cultures. If the temperature reaction is only moderate, not over 40.5°C., an injection of the bacteria from 40 cc. of culture is given on the following day. If the reaction from this is only moderate, the dose is again doubled on the following day and the bacteria from 80 cc. of culture are injected. As before stated, so far as pneumococci are concerned it is difficult to regulate the dosage entirely by the febrile reaction obtained. If the reaction is very severe, of course the dose is made smaller than those mentioned. On the other hand, even though the reaction is very slight, we do not advise giving more than the amounts stated. 6 days after the last injection another specimen of blood is obtained for testing. If the serum is of the standard strength, bleeding can now be done. If it is still too weak, a second

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<sup>34</sup> Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.



series of injections of living bacteria is made. These injections should consist of the bacteria from 100 cc., 150 cc., and 200 cc. of broth on the 3 days respectively. Again, the size of the doses may need to be somewhat modified, on account of the severity or lack of febrile reaction. Certain horses may require still further injections of live cultures but this is exceptional. In any case, we think the injections should be made in series of three, given on successive days, with 7 day intervals between each series, and from our present standpoint we believe that the size of the injection should never be greater than the bacteria from 300 to 400 cc. of broth. Following the bleeding, it is well to allow the horse to remain quiet for 3 or 4 days. Then a series of three injections of living cultures, 50, 80, and 100 cc., is again given. After a week the serum is again tested and if of standard strength bleeding may again be done on the 10th day following the last injection.

#### CONCLUSIONS.

In the production of immune serum for therapeutic purposes strict attention must be paid to the immunological specificity of the bacteria used for immunization. At present the only serum of which the therapeutic value has been proven is that effective against Type I pneumococcus infection. This serum should have agglutinating power for Type I pneumococcus and should have the power of protecting mice against large amounts of virulent culture. Experiments have shown that for producing the primary immunity most rapidly several series of small doses of dead cultures should be given, the injections being made daily for 6 to 7 days, followed by a week in which no injections are made. To produce the highest type of immunity probably living organisms are required. These should be given in moderate doses daily for 3 days, with an interval of a week between each series of injections. By following accurately the methods described, horses may be made to produce rapidly a high grade of specific serum. The observations so far made indicate the importance of employing small doses of culture frequently repeated in this form of immunization.



## STUDIES ON ANTIBLASTIC IMMUNITY.\*

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The following studies were undertaken with the purpose of throwing more light, if possible, on the influence exerted by immune serum on the metabolic activities of bacteria. It has been shown that under certain experimental conditions immune serum possesses the property of inhibiting or markedly retarding the metabolic functions of various microorganisms. Bacterial metabolic processes are generally considered to be enzymotic in nature. It is not known whether they take place primarily at the surface of the bacterial cell or within the cell body. It is probable, however, that exogenous metabolism occurs to a considerable extent in preparing nutrient material for absorption.

Evidence for this may be found in the studies by Cole<sup>1</sup> on the formation of methemoglobin by the pneumococcus. The property of immune serum which enables it to inhibit the metabolic activities of bacteria is considered to act at the surface of the bacterial cell and to be antienzymotic in nature. The term antiblastic immunity has been applied as descriptive of the phenomenon.

Von Dungern<sup>2</sup> has shown that the liquefaction of gelatin by *Staphylococcus pyogenes aureus* is inhibited by antistaphylococcus immune serum; Ghéorghiewsky<sup>3</sup> showed that immune serum inhibits pigment formation by *B. pyocyaneus*; and Ascoli<sup>4</sup> considered that the action of antianthrax serum was in part dependent upon the inhibition of capsule formation by *B. anthracis* with consequent suppression of the metabolic activities of the organism. More recently Dochez and Avery<sup>5</sup> have shown that antipneumococcus immune serum retards the growth of the pneumococcus and inhibits or markedly delays the fermentation of inulin and the splitting of protein by the pneumococcus.

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\* This work was done under the tenure of a William O. Moseley Travelling Fellowship from Harvard University.

<sup>1</sup> Cole, R., *J. Exp. Med.*, 1914, xx, 363.

<sup>2</sup> von Dungern, *Centr. Bakteriolog., 1te Abt.*, 1898, xxiv, 710.

<sup>3</sup> Ghéorghiewsky, *Ann. Inst. Pasteur*, 1899, xiii, 298.

<sup>4</sup> Ascoli, A., *Centr. Bakteriolog., 1te Abt., Orig.*, 1908, xlvi, 178.

<sup>5</sup> Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.

## EXPERIMENTAL.

The majority of the experiments reported below deal with the antiblastic properties of antipneumococcus serum. A few experiments have been done with antistaphylococcus serum.

*Inhibition of the Metabolic Activities of the Pneumococcus by Antipneumococcus Immune Serum.*

Characteristic metabolic functions of the pneumococcus are the fermentation of inulin and litmus milk, the splitting of protein, and the transformation of oxyhemoglobin to methemoglobin. The influence which immune serum exerts on these functions of the pneumococcus has been studied. The experiments of Dochez and Avery have been repeated and extended and their experimental results confirmed in that under certain conditions antipneumococcus serum has been found to possess the property of inhibiting or retarding the metabolic activities of pneumococci. The conclusions drawn from our experiments as to the mechanism of this inhibitory property of antipneumococcus serum, however, differ from those of Dochez and Avery.

*Experiment 1. Inhibition of the Growth of the Pneumococcus by Antipneumococcus Serum.*—In Table I is shown one of a series of experiments on the rate of growth of the pneumococcus in homologous and heterologous antipneumococcus serum and in normal horse serum. The amount of growth was determined by the plate method.

This experiment shows that an apparent inhibition of growth occurs in homologous antipneumococcus serum as compared with that in normal serum during a period of 3 hours, that a retardation of growth occurs up to 6 hours, but that by 24 hours an abundant growth is present in the immune serum as well as in the normal serum. It furthermore appears that there is some retardation of growth of Type I pneumococcus by the heterologous immune serum, a phenomenon which does not occur with Type II pneumococcus. These results agree with those of Dochez and Avery. It is felt, however, that the interpretation of such results as indicative of actual retardation of growth is open to serious objection. It is clear that the apparent inhibition of growth by homologous serum must in large part

TABLE I.

*Inhibition of the Growth of the Pneumococcus by Antipneumococcus Serum.*

Culture 0.00000001 cc.	Serum 0.2 cc.	No. of colonies.			
		Imme- diately.	After 3 hrs.	After 6 hrs.	After 24 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	61	54	80	Confluent.
" " I	Antipneumococcus Serum Type II	50	45	12,000	"
" " I	Normal horse serum.	37	284	22,000	"
Pneumococcus Type II	Antipneumococcus Serum Type I	31	69	6,200	Confluent.
" " II	Antipneumococcus Serum Type II	43	42	1,860	"
" " II	Normal horse serum.	29	59	7,750	"

be due to agglutination. Furthermore, it is well known that during the first hours of growth of the pneumococcus chain formation occurs to a considerable extent. This is a variable phenomenon which is difficult to control. For these reasons it seems hazardous to conclude from the experiments that immune serum actually retards the growth of the pneumococcus and quite as probable that the inhibition indicated by the colony counts is more apparent than real.

*Experiment 2. Inhibition of the Fermentation of Inulin and Litmus Milk by Antipneumococcus Serum.*—The power of antipneumococcus serum to inhibit the fermentation of inulin and litmus milk by the pneumococcus has been tested in a considerable series of experiments, one of which is shown in Table II.

This experiment demonstrates that the fermentation of litmus milk by the pneumococcus is markedly delayed in the presence of the homologous immune serum. The fermentation of inulin is retarded by antipneumococcus serum in a similar manner. In the tubes containing the homologous immune serum the organisms grow in agglutinated masses at the bottom of the tube, while in the tubes containing the heterologous or normal serum they grow diffusely. To demonstrate clearly inhibition of fermentation it was found necessary to use extremely small amounts of pneumococcus culture (not more than 0.00001 cc.) in inoculating the media. With the use of

TABLE II.

*Inhibition of the Fermentation of Litmus Milk by Antipneumococcus Serum.*

Litmus milk 4 cc. +		Incubation at 37°C.			
Culture 0.000001 cc.	Serum 1 cc.	24 hrs.	48 hrs.	72 hrs.	5 days.
Pneumococcus Type I	Antipneumococcus Serum Type I	—	—	±	+
“ “ I	“ “ “ II	++	++	++	++
“ “ I	Normal horse serum.	++	++	++	++
Pneumococcus Type II	Antipneumococcus Serum Type I	++	++	++	++
“ “ II	“ “ “ II	—	+	±	+
“ “ II	Normal horse serum.	++	++	++	++

++ indicates complete acidification and coagulation; ++, acid and incomplete coagulation; +, acid and beginning coagulation; ±, acid and no coagulation; ±, slight acidification and no coagulation; —, no acidification or coagulation.

larger amounts\* retardation of fermentation was very slight or did not occur at all. In a series of experiments to determine the maximum amount of culture that could be used satisfactorily in demonstrating the antiblastic action of immune serum in preventing the fermentation of inulin, it was frequently noted that in the immediate vicinity of the agglutinated pneumococci at the bottom of the culture tube there was acidification of the medium indicated by change of the litmus indicator to red, while the upper portion of the medium remained unchanged. This observation suggested the possibility that the apparent antiblastic action of antipneumococcus serum was in reality due merely to agglutination of the organisms at the bottom of the culture tube and their consequent inability to come into intimate contact with the medium as a whole, this not being the case in the presence of heterologous immune or normal serum. If this is so, to conclude from such experiments as those cited above that immune serum possesses a specific antiblastic action would hardly be justified. Further experiments on this point are given below.

*Experiment 3. Inhibition of Methemoglobin Formation by Antipneumococcus Serum.*—The transformation of oxyhemoglobin to methemoglobin is a characteristic metabolic function of the pneumococcus which lends itself readily to *in vitro* experiments. A large series of experiments has been done in order to determine under what conditions antipneumococcus serum will inhibit this

activity of the pneumococcus. In general, the results have been similar to those obtained in the case of litmus milk and inulin. To demonstrate inhibition, it was found essential to use very small amounts of pneumococci in inoculating the hemoglobin solution-serum mixtures and to allow the cultures to incubate 24 hours. One of these experiments is shown in Table III, which demonstrates that under these conditions homologous antipneumococcus serum inhibits the transformation of oxyhemoglobin to methemoglobin by the pneumococcus. Heterologous immune serum and normal horse serum possessed no inhibitory properties.

TABLE III.

*Inhibition of Methemoglobin Formation by Antipneumococcus Serum.*

Hemoglobin solution* 1 cc. +		Methemoglobin formation.	
Culture 0.00001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	—	—
“ “ I	“ “ II	++	++
“ “ I	Normal horse serum.	++	++
Pneumococcus Type II	Antipneumococcus Serum Type I	++	++
“ “ II	“ “ II	—	±
“ “ II	Normal horse serum.	++	++

++ indicates complete transformation of oxyhemoglobin to methemoglobin; ±, slight methemoglobin formation; —, no methemoglobin formation.

\* Hemoglobin solution = 2 cc. of a 5 per cent suspension of washed rabbit corpuscles + 8 cc. of distilled water + 30 cc. of 0.85 per cent salt solution.

With the use of larger amounts of culture it was found that the formation of methemoglobin was not inhibited by immune serum, but took place rapidly in the immediate vicinity of the agglutinated pneumococci and gradually diffused upward throughout the whole medium. These results, as in the case of inulin, suggested that the apparent inhibition which occurs when minute amounts of culture are used is due to inability of the agglutinated pneumococci to come into intimate contact with the medium as a whole.

*Experiment 4. Inhibition of the Proteolytic Functions of the Pneumococcus by Antipneumococcus Serum.*—The growth of pneumococcus in a serum broth medium is attended by an increase in the amino-acid content of the medium. Dochez and Avery<sup>5</sup> believe it probable that the pneumococcus effects a splitting of pro-

tein before absorption and that the increase in amino-acid content of the medium represents the excess of protein split over that used up in the process of growth. If this hypothesis is accepted, the increase in amino-acid produced by the growth of the pneumococcus might serve as a measure of the proteolytic activity of the pneumococcus in a given culture, the amount of increase being directly proportional to the total amount split. The possibility should be pointed out, however, that the amount of amino-acid increase might be inversely proportional to the metabolic activities of the pneumococcus, a small amino-acid increase indicating a greater utilization of the total amount of protein split, and *vice versa*.

The experiment recorded in Table IV shows the increase in amino-acid nitrogen when the pneumococcus was grown in broth containing antipneumococcus serum as compared with the increase in the presence of normal horse serum. The increase in amino-acid was determined by the method of Van Slyke.<sup>6</sup>

TABLE IV.

*Inhibition of the Proteolytic Activities of the Pneumococcus by Antipneumococcus Serum.*

Broth 8 cc. +		Increase in amino nitrogen per cc. after 48 hrs. at 37° C.
Culture 0.000002 cc.	Serum 2 cc.	
Pneumococcus Type I	Antipneumococcus Serum Type I	mg. 0.031
“ “ I	“ “ II	0.103
“ “ I	Normal horse serum.	0.106

If the increase in amino nitrogen content of the medium is a direct measure of the proteolytic activities of the pneumococcus, it is apparent that the homologous antipneumococcus serum exerted a marked inhibitory action on this metabolic function of the pneumococcus. Since the pneumococcus growth in the tube containing homologous immune serum was in agglutinated masses at the bottom of the tube in contradistinction to the diffuse growth in the other tubes, it seemed possible that this factor might be responsible for the apparent inhibition of proteolytic activity rather than any true antienzymotic property of the serum. A further experiment to determine the influence of this factor is presented below.

*Experiment 5. Antiblastic Properties of Exhausted Immune Serum.*—A series of experiments was done to determine whether immune serum exhausted of its

<sup>6</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 121; 1915, xxiii, 407.



agglutinin and precipitin content by repeated exposure to killed pneumococci still retained any demonstrable antiblastic properties. In Table V is shown one of these experiments from which it will be seen that immune serum exhausted of its agglutinin and precipitin content possesses no power to inhibit the formation of methemoglobin by the pneumococcus. Identical results have been obtained with respect to the fermentation of inulin and litmus milk. From these experiments it is evident that the specific antiblastic action of immune serum, if such exists, is removed from the serum at the same time that the agglutinins and precipitins are removed by treatment with dead pneumococci.

TABLE V.

*Antiblastic Properties of Exhausted Antipneumococcus Serum.*

Hemoglobin solution 1 cc. +		Methemoglobin formation.	
Culture 0.00001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.
Pneumococcus Type I	Antipneumococcus Serum Type I	—	—
“ “ I	Exhausted “ “ “ I	++	++
Pneumococcus Type II	Antipneumococcus Serum Type II	—	±
“ “ II	Exhausted “ “ “ II	++	++

*Experiment 6. Relation of Agglutinins to Antiblastic Immunity.*—From the results obtained in the foregoing experiments the suggestion arose that the apparent antiblastic action of antipneumococcus serum was in some way closely related to agglutination of the bacteria. The following experiment was done to determine more accurately this relationship. Equal parts of pneumococcus cultures and increasing serum dilutions were mixed in small tubes and incubated for 2 hours at 37°C. 1 cc. of hemoglobin solution was then carefully added to each tube so as not to disturb the agglutinated pneumococci and the tubes were incubated for 1 hour at 37°C. The results are shown in Table VI.

This experiment shows that the antiblastic action of the serum bears a definite relationship to the degree of agglutination of the pneumococci. In the tube containing the 1:25 serum dilution agglutination was complete, the pneumococci having entirely settled to the bottom of the tube, and complete inhibition of methemoglobin formation occurred. With increasing dilutions of serum agglutination and sedimentation were progressively less complete and formation of methemoglobin rapidly took place in the immediate neighborhood of the agglutinated pneumococci, the upper portion of the medium

TABLE VI.  
*Relation of Antiblastic Immunity to Agglutination.*

Culture 0.5 cc.	Serum 0.5 cc.	Agglutination after 2 hrs. at 37° C.		Methemoglobin formation after 1 hr. at 37° C.
Pneumococcus Type I	Antipneumococcus Serum Type I (1:25)	++	1 cc. of hemoglobin solution added to each tube.	-
" " I	Antipneumococcus Serum Type I (1:50)	++		-
" " I	Antipneumococcus Serum Type I (1:100)	+		++
" " I	Antipneumococcus Serum Type I (1:200)	±		-
" " I	Antipneumococcus Serum Type I (1:400)	-		++
" " I	Antipneumococcus Serum Type II (1:25)	-		++
" " I	Normal horse serum (1:25)	-		++

± indicates methemoglobin formation in the immediate vicinity of the agglutinated pneumococci, the upper portion of the medium remaining unchanged.

from which the bacteria had settled out remaining unchanged. This experiment indicates that the antiblastic property of antipneumococcus serum probably depends in large measure, if not entirely, upon its ability to agglutinate the pneumococci and thereby prevent their coming into intimate contact with the medium as a whole.

By growing pneumococci in a serum-hemoglobin solution agar medium, agglutination of the pneumococci may be prevented. Under these conditions antipneumococcus serum was found not to inhibit methemoglobin formation. An objection may be raised to these experiments, however, because it is not certain that in a solid medium the immune serum is in sufficient concentration in the immediate vicinity of the pneumococcus colonies to exert a demonstrable antiblastic action.

*Experiments 7 and 8. Relation of Antiblastic Immunity to the Ability of Bacteria to Come into Intimate Contact with the Medium.*—It seemed probable from many of the previous experiments that the antiblastic action of antipneumococcus serum was due to inability of the agglutinated bacteria to come into sufficiently

intimate contact with the medium as a whole to enable them to produce the characteristic changes in the medium. Under these conditions the metabolic activities of the pneumococci could exert their influence only in the immediate vicinity of the agglutinated bacteria and complete change of the medium could take place only as gradual diffusion occurred. This is exactly what did occur when moderately large amounts of pneumococci were used, the reaction in the presence of homologous immune serum being merely retarded rather than completely inhibited. When minute amounts of pneumococci were used growth had probably ceased before diffusion could take place and apparently complete inhibition resulted.

In order to obviate this difficulty, two sets of experiments have been carried out. The first consisted in running a parallel series of tubes, one of which was shaken, the other not. The second group of experiments consisted in a parallel series of cultures, one in test-tubes, the other in small flasks so that the agglutinated bacteria would be brought into much more intimate contact with the medium as a whole, which was in a thin layer at the bottom of the flasks, than could possibly occur in test-tube cultures. The effect of shaking is well demonstrated in the experiment shown in Table VII.

TABLE VII.

*Effect of Shaking on the Inhibition of Methemoglobin Formation by Antipneumococcus Serum.*

2 hrs. at 37°C.			Incubation at 37°C.				
Culture 0.5 cc.	Serum 0.5 cc.			10 min.	30 min.	1 hr.	Shaken 10 min.
Pneumococcus Type I	Antipneumococcus Serum Type I	1 cc. of hemoglobin solution in each tube.	Unshaken.	—	—	—	++
“ “ I	Antipneumococcus Serum Type I		Shaken.	++	++	++	

Suitable control tubes showed that normal horse serum did not inhibit methemoglobin formation and that shaking in itself did not change oxyhemoglobin to methemoglobin. While complete inhibition of methemoglobin formation took place in the unshaken tube in the presence of homologous antipneumococcus serum, in the tube that was shaken so that the agglutinated clumps of bacteria were more or less broken up and diffused throughout the medium no inhibition occurred.

Further experiments illustrating this point were made with two strains of *Streptococcus viridans* which, like the pneumococcus, transforms oxyhemoglobin to methemoglobin. Strain 1 normally grew diffusely in serum-free bouillon, but like many streptococci grew in granular clumps at the bottom of the culture tube in the presence of serum. By growing this streptococcus in tubes containing antipneumococcus serum, it was possible to inhibit completely for 24 hours the formation of methemoglobin as shown in Table VIII, demonstrable methemoglobin being present only after gradual diffusion of the medium took place.

TABLE VIII.

*Inhibition of Methemoglobin Formation by Streptococcus viridans by Antipneumococcus Serum.*

Hemoglobin solution 1 cc. +			Methemoglobin formation.		Character of growth.
Culture 0.00001 cc.	Serum 1 cc.		After 24 hrs.	After 48 hrs.	
<i>Streptococcus viridans</i> 1	Antipneumococcus	Serum	—	+	Sediment.
“ “ 1	Type I Antipneumococcus	Serum	—	+	“
“ “ 1	Type II Normal horse serum.		±	++	“ and dif- fuse.
“ “ 1	Bouillon 1 cc.		++	++	Diffuse.

This inhibition cannot be attributed to any specific immunity principle in the serum and is more probably brought about by the growth of the streptococci at the bottom of the culture tube and their consequent inability to act on the medium as a whole.

*Streptococcus viridans* 2 normally grew as a sediment in bouillon and produced methemoglobin slowly as the growth gradually extended up the sides of the tube and diffusion of the medium took place. By shaking a culture of *Streptococcus viridans* 2 so that the organisms were in continual intimate contact with the whole medium it was found that the transformation of oxyhemoglobin to methemoglobin took place rapidly and was complete by the end of 6 hours,

at which time the unshaken control tubes failed to show any trace of methemoglobin (Table IX).

TABLE IX.

*Effect of Shaking on Methemoglobin Formation by Streptococcus viridans.*

Materials used.	Methemoglobin formation after incubation at 37°C.	
	3 hrs.	6 hrs.
<i>Streptococcus viridans</i> 2, 0.5 cc. + hemoglobin solution 1 cc. + bouillon 1 cc. (shaken).....	±	++
<i>Streptococcus viridans</i> 2, 0.5 cc. + hemoglobin solution 1 cc. + bouillon 1 cc. (unshaken).....	—	—
Hemoglobin solution 1 cc. + bouillon 1.5 cc. (shaken).....	—	—
“ “ 1 “ + “ 1.5 “ (unshaken).....	—	—

It is evident from this experiment that the rapid formation of methemoglobin by this streptococcus depended upon its ability to grow in intimate contact with the whole medium.

Comparison of the inhibitory power of immune serum in parallel series of test-tube cultures and flask cultures confirmed the results of the shaking experiments. It was found that while marked retardation of metabolic function as measured by the change in the medium occurred in the test-tube in the presence of homologous immune serum, no inhibition occurred in the flask cultures in which the agglutinated bacteria were able to come into intimate contact with the medium as a whole. This is clearly shown in the experiments recorded in Table X.

From these experiments it is evident that antipneumococcus serum exerted no inhibitory action on the fermentation of litmus milk or on the splitting of protein by the pneumococcus when the organisms were grown in flask cultures, in striking contrast with the results in the test-tube cultures. Identical results were obtained with respect to the fermentation of inulin.

These two groups of experiments seem to throw considerable doubt on the theory that antipneumococcus serum possesses the property of inhibiting the metabolic activities of the pneumococcus by virtue of a specific antiblastic immunity principle. They rather indicate

TABLE X.

*Relation of the Inhibition of the Metabolic Activities of the Pneumococcus by Antipneumococcus Serum to the Ability of the Pneumococcus to Act on the Medium as a Whole.*  
*Fermentation of Litmus Milk.*

Litmus milk 4 cc. +		Test-tube cultures.			Flask cultures.		
Culture 0.000001 cc.	Serum 1 cc.	After 24 hrs.	After 48 hrs.	After 72 hrs.	After 24 hrs.	After 48 hrs.	After 72 hrs.
Pneumococcus Type II	Antipneumococcus Type I Serum	++	++	++	++	++	++
" " II	Antipneumococcus Type II Serum	-	+	=	++	++	++
" " II	Normal horse serum.	++	++	++	++	++	++

*Digestion of Protein.*

Broth 8 cc. +		Increase in amino nitrogen per cc. after 48 hrs. at 37°C.	
Culture 0.000002 cc.	Serum 2 cc.	Test-tube cultures.	Flask cultures.
		mg.	mg.
Pneumococcus Type I	Antipneumococcus Serum Type I	0.031	0.169
" " I	" " " II	0.103	0.120
" " I	Normal horse serum.	0.106	0.154

that the inhibition which occurs under certain experimental conditions is in reality due to agglutination of the pneumococci by the immune serum and their consequent inability to come into intimate contact with the medium as a whole.

*Inhibition of the Metabolic Activities of Staphylococcus pyogenes aureus by Antistaphylococcus Serum.*

Characteristic metabolic functions of *Staphylococcus pyogenes aureus* are the liquefaction of gelatin, the formation of a golden pigment, and the transformation of oxyhemoglobin to a magenta-colored unstable compound, which is probably reduced hemoglobin.

A limited series of experiments has been carried out to determine the ability of antistaphylococcus serum to inhibit or retard these metabolic activities of *Staphylococcus pyogenes aureus*. The staphylococcus used in the experiments was obtained by blood culture from a case of staphylococcus septicemia and an autologous immune serum was prepared by the immunization of a rabbit with this staphylococcus.

*Experiment 9. Inhibition of the Liquefaction of Gelatin by Antistaphylococcus Serum.*—The liquefaction of gelatin is a characteristic metabolic function of *Staphylococcus pyogenes aureus*. In a series of experiments to determine the inhibitory action exerted by antistaphylococcus serum on this activity of the staphylococcus it was found that complete inhibition occurred. One of these experiments is shown in Table XI. Incubation of the culture tubes was carried out at 20°C. so that no agglutination of the staphylococci would occur. It should be noted that in the tube containing antistaphylococcus serum there was no apparent inhibition of growth of the staphylococci as compared with that in the control tube. In other words, although the liquefaction of gelatin was completely inhibited, the immune serum exerted no apparent antagonistic action towards those metabolic activities of the staphylococcus which are essential to its growth and multiplication.

TABLE XI.

*Inhibition of the Liquefaction of Gelatin by Antistaphylococcus Serum.*

Gelatin 4 cc. +		Liquefaction of gelatin after incubation at 20°C.		
Culture 0.000001 cc.	Serum 1 cc.	3 days.	6 days.	9 days.
<i>Staphylococcus pyogenes aureus</i> .	Antistaphylococcus serum.	—	—	—
“ “ “	Normal rabbit serum.	++	++	++

*Experiment 10. Inhibition of Pigment Formation by Antistaphylococcus Serum.*—When staphylococci were grown on the surface of serum agar slants and in serum agar shake cultures, it was found that antistaphylococcus serum exerted no inhibitory effect on pigment formation by the organisms. It was felt that under these conditions the serum might not be in sufficient concentration in the immediate vicinity of the staphylococcus colonies to exhibit any demonstrable antiblastic action. In order to obviate this objection as far as possible, serum agar plates were prepared (serum 1 part to plain agar 4 parts) and a thin layer of serum was spread over the surface of the agar which was then streaked with a loop of

staphylococcus culture. The plates were sealed to prevent evaporation of the serum as far as possible and incubated at room temperature. Under these conditions the growing staphylococcus colonies were in intimate contact with the serum. It was found that antistaphylococcus serum definitely retarded the formation of pigment by *Staphylococcus pyogenes aureus* (Table XII), although it exerted no apparent inhibitory action on the rate of growth of the bacteria, as compared with the growth in the presence of normal serum.

TABLE XII.

*Inhibition of Pigment Formation by Antistaphylococcus Serum.*

Materials used.	Pigment formation.		
	After 24 hrs.	After 36 hrs.	After 48 hrs.
Antistaphylococcus serum agar + <i>Staphylococcus pyogenes aureus</i> .....	—	—	±
Normal serum agar + <i>Staphylococcus pyogenes aureus</i> .....	±	+	++

This inhibition of pigment formation by antistaphylococcus serum must be attributed to some definite antagonistic property of the serum toward this function of the staphylococcus. It should not be concluded, however, from this inhibitory phenomenon that the antagonistic action of the serum is definitely antienzymotic in nature, for it is not definitely known that pigment formation by *Staphylococcus pyogenes aureus* is caused by enzyme action. Certainly the formation of pigment is not essential to the life and growth of the staphylococcus, for it does not occur during the early stages of growth and appears only after the staphylococcus colonies are well developed and multiplication probably has in large measure ceased. Further experiments are necessary to establish the exact nature of this inhibitory action of antistaphylococcus serum.

*Experiment 11. Inhibition of the Reduction of Oxyhemoglobin by Antistaphylococcus Serum.*—To determine the inhibitory effect of antistaphylococcus serum on the reduction of oxyhemoglobin by *Staphylococcus pyogenes aureus*, two series of experiments were made, one with the use of a 5 per cent suspension of washed rabbit corpuscles, the other with a solution of hemoglobin prepared from rabbit corpuscles as in the pneumococcus experiments. A typical experiment is shown in Table XIII.



TABLE XIII.

*Inhibition of the Reduction of Oxyhemoglobin by Antistaphylococcus Serum.*

Suspension of rabbit corpuscles 1 cc. +		Reduction of oxyhemoglobin after incubation at 37°C.	
Culture 0.000001 cc.	Serum 0.5 cc.	24 hrs.	48 hrs.
<i>Staphylococcus pyogenes aureus.</i>	Antistaphylococcus serum.	++	++
" " "	Normal rabbit "	++	++
" —	" " "	—	—

Hemoglobin solution 1 cc. +		Reduction of oxyhemoglobin after incubation at 37°C.	
Culture 0.000001 cc.	Serum 0.5 cc.	24 hrs.	48 hrs.
<i>Staphylococcus pyogenes aureus.</i>	Antistaphylococcus serum.	—	±
" " "	Normal rabbit "	++	++
" —	" " "	—	—

It was found that the reduction of oxyhemoglobin was not inhibited by antistaphylococcus serum when rabbit corpuscles were used, but that inhibition occurred when a hemoglobin solution was substituted for the corpuscle suspension. This difference is readily explained on the basis of the results obtained in the pneumococcus experiments. In the tubes containing rabbit corpuscles the blood cells rapidly settled to the bottom of the tube where they were in intimate contact with the agglutinated staphylococci, and were therefore in a position to be acted upon by the organisms. In the tubes containing hemoglobin solution, however, the agglutinated staphylococci were not in intimate contact with the hemoglobin held in solution throughout the medium and consequently were unable to act upon it within the time limits of the experiment. When the agglutinated clumps of staphylococci were broken up and dispersed throughout the medium by shaking, the hemoglobin solution was rapidly reduced. From these experiments it is evident that antistaphylococcus serum possesses no specific antiblastic property which enables it to inhibit the reduction of oxyhemoglobin by *Staphylococcus pyogenes aureus*.

## DISCUSSION.

The series of experiments reported in this paper were undertaken in an attempt to explain the mechanism by which immune serum is able to retard or inhibit the metabolic activities of bacteria. This property of immune serum has been attributed by previous investigators to an antienzymotic or antiblastic action of the serum which exerts itself at the point of contact of the bacterial cell with its environment. While it has been clearly demonstrated in confirmation of the work of other observers that under certain experimental conditions immune serum does exert an apparent inhibitory action on the nutritional and certain other metabolic processes of bacteria, the facts brought out as the experiments progressed have made it seem probable that the inhibitory action exerted by antipneumococcus serum is not due to a specific antienzymotic or antiblastic property of the serum, but rather to its agglutinating properties. It is well recognized that many bacteria grow readily in homologous immune serum, provided the serum does not possess definite bactericidal properties, a fact which in itself would tend to throw some doubt on the validity of the antiblastic theory as applicable to all immune sera, since metabolic activity is considered to be essential to bacterial nutrition and growth. Bacteria when cultivated in media containing immune serum grow in agglutinated clumps at the bottom of the culture tube. Under these conditions they do not come into intimate contact with the whole medium and are unable to bring about changes rapidly throughout the medium. Presumably the metabolic activities which bacteria carry on in the process of growth exert their influence on the medium only in the immediate vicinity of the agglutinated organisms during the early stages of growth, and only as gradual diffusion of the medium takes place are the bacteria able to act upon the whole medium in sufficient degree to bring about those changes which indicate that bacterial metabolism is taking place. That these considerations hold with respect to the influence exerted by antipneumococcus serum on the metabolic activities of the pneumococcus has been demonstrated in the experiments reported above, in which it has been shown that the metabolic activities of the pneumococcus do take place in the immediate vicinity of the agglutinated bacteria in spite of the

presence of immune serum; that the degree of apparent inhibition which antipneumococcus serum exerts upon the metabolic activities of the pneumococcus depends upon the extent of agglutination of the organisms; that antipneumococcus serum exhausted of its agglutinin content possesses no antiblastic properties; that antipneumococcus serum possesses the property of apparently inhibiting the metabolic activities of entirely unrelated bacteria provided those bacteria grow in sedimented clumps in the presence of the serum; and that when pneumococci are grown in the presence of antipneumococcus serum under conditions that enable them to come into intimate contact with the whole medium, no inhibition of metabolic activity takes place.

A limited number of experiments with antistaphylococcus serum has confirmed the results obtained with antipneumococcus serum in as far as the inhibitory effect of the serum on the reduction of oxyhemoglobin by the staphylococcus is concerned. On the other hand, antistaphylococcus serum has been found to inhibit or markedly retard the formation of pigment and the liquefaction of gelatin by *Staphylococcus pyogenes aureus*. Under the conditions of the experiments agglutination of the bacteria did not occur and therefore could not have been a factor in causing the inhibition. It is to be noted that neither pigment formation nor the liquefaction of gelatin is an essential process in the nutrition of the staphylococcus and that these phenomena take place in demonstrable amount only after the staphylococcus colonies have become well developed. It is felt that further experimental work is necessary to determine the exact mechanism of the inhibitory action of antistaphylococcus serum on these functions of the staphylococcus before the conclusion can be safely drawn that it is of an antienzymotic nature. The outstanding fact that staphylococci as well as pneumococci grow rapidly and abundantly in the homologous immune serum is sufficient in itself to indicate that the immune serum possesses no properties in demonstrable amount antagonistic to those bacterial metabolic activities essential to the growth and multiplication of the bacteria.

## CONCLUSIONS.

Antipneumococcus serum under certain conditions apparently inhibits or retards the metabolic activities of the homologous pneumococcus.

Antipneumococcus serum exhausted of its agglutinin content possesses no inhibitory properties.

The degree of inhibitory action of antipneumococcus serum parallels its agglutinating power.

No evidence has been found to indicate that the inhibition of the metabolic activities of the pneumococcus by antipneumococcus serum is due to a specific antienzymotic property of the serum.

The evidence which has been obtained indicates that the apparent inhibition of metabolic activity that occurs under certain conditions is due to agglutination of the pneumococci by the antipneumococcus serum and their consequent inability to grow in intimate contact with the whole medium.

Antistaphylococcus serum inhibits the liquefaction of gelatin and the formation of pigment by *Staphylococcus pyogenes aureus*. Further experimentation is necessary to determine the mechanism of this inhibitory action.

Antistaphylococcus serum does not inhibit those metabolic activities of *Staphylococcus pyogenes aureus* essential to the growth and multiplication of the organisms.

## FURTHER STUDIES IN EXPERIMENTAL ATHERO-SCLEROSIS.

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PLATES 40 TO 43.

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Several years ago, the writer<sup>1</sup> reported some experiments on dogs as a contribution to the study of experimental atherosclerosis. Since that preliminary report, the experiments have been continued and though much of these very elusive problems is still far from being clear, it is thought best to report some of the results of further study. It may be well to summarize briefly the contents of the article just referred to. Infectious disease has frequently been insisted upon as an important etiological factor in atherosclerosis. In order to test this, dogs were infected with pure cultures of various pathogenic bacteria and also with bacterial toxins, by means of injections into the jugular vein. The animals were kept in a state of mild chronic sepsis for periods ranging from 4 to 7 months. The results, as far as the blood vessels were concerned, were entirely negative. Lead and nicotine also gave negative results. General overexertion maintained for long periods by means of a treadmill, both with and without the hypertension produced by the injection of large doses of adrenalin, also proved without effect. The parenteral methods were then abandoned and feeding experiments were taken up. Some attempts were made with cottonseed-oil, which, however, proved too toxic. Then two dogs were fed daily with pure cholesterol in half-gram doses enclosed in capsules. These two dogs died after 1½ and 2½ months respectively. In the former publication positive results are claimed for these two experiments, because a number of whitish yellowish streaks, very slightly raised above the level of the intima, was found in the aortas. In the light of later experience these can no longer be considered as positive results. The doses of cholesterol were too small, dogs eliminate cholesterol more rapidly and completely than rabbits, and under these conditions 1½ and 2½ months would appear altogether too short a time for the development of so insidious and chronic a lesion as atherosclerosis. Moreover, experience has shown that these insignificant ridges and patches are found fairly regularly in the aorta of young

<sup>1</sup> Adler, I., Studies in experimental atherosclerosis. A preliminary report, *J. Exp. Med.*, 1914, xx, 93.

and healthy individuals both canine and human. There will be occasion to speak of this more in detail presently. It is not necessary to recount here all the varieties of experimentation that were attempted. It is important however to mention two dogs that received, besides their usual food of bread and meat, dilute hydrochloric acid up to sixty drops daily for 2 months and 3 months respectively. Both these dogs showed very definite and unmistakable arteriosclerotic patches in various portions of the aorta and in the one case, especially well marked in the lowest portion of the abdominal aorta. There were no traces of ulceration or calcification. Microscopically these patches showed the typical picture of arteriosclerosis, the details of which need not be gone into, except to mention the fact that cholesterol, in its free state, was abundantly present. That these findings could not be accepted unhesitatingly as proving hydrochloric acid to be an important factor in the etiology of arteriosclerosis is obvious. Two experiments are altogether insufficient to clinch so important a conclusion. The time during which the hydrochloric acid feeding was continued was too short, and more important than everything else, the age of the animals could not be ascertained and both dogs were in all probability rather old. It is easily possible, therefore, that the lesions found were not due to experimentation but were spontaneous, for it can no longer be doubted, though it is perhaps not generally recognized, that arteriosclerosis, closely analogous to the human, occurs spontaneously in old and debilitated dogs. It was necessary therefore to continue these acid experiments on a little broader basis.

The writer is still of the opinion that if it be possible to produce atherosclerosis, analogous to, or identical with the human type, experimentally and with reasonable certainty, the methods employed should give positive results not only in rabbits, but with a fair constancy in all animals that are subject to spontaneous or typical senile atherosclerosis. Unfortunately animal pathology has been more or less neglected in this respect, and it is possible that when more is known of vascular disease in the animal kingdom, more favorable subjects for the experimental study of atherosclerosis will be found. Until then, however, dogs would seem sufficiently well adapted for this class of experiments. They are omnivorous as the human; the structure of their blood vessels, especially of the aorta, differs not so very greatly from the structure of human arteries; and while they are subject to pronounced senile atherosclerosis, the lesions are not obscured and rendered uncertain by such forms of median necrosis as occur spontaneously in so large a percentage of rabbits. It is the writer's firm conviction, therefore, that the following conditions

should be fulfilled before it can be justly claimed that a genuine atherosclerosis has been artificially produced by experiment. (1) The gross lesions must be closely analogous, if not quite identical in every microscopic detail with the human arteriosclerosis. (2) These positive results must be so constant and regular that it is reasonably to be expected that every experiment will give a positive result, with perhaps only an occasional failure under exceptional conditions. Definite conclusions from apparently positive findings in only one or two animals are not permissible. The number of positive results must be sufficiently large, though an exception here and there may occur. In man also there occur instances, from the celebrated case of Harvey's down to modern times, where persons can live even to extreme old age, apparently immune to arteriosclerosis, their arteries remaining soft and elastic up to their death. (3) In order to avoid the probability of the lesions being due to spontaneous degeneration, the experiments should all be done on young animals. It might be objected to this that the youthful organism is perhaps possessed of powers of resistance and repair which are materially diminished or lost in older individuals and that therefore experiments on very young animals would not be likely to give positive results by the method in question even if they fulfilled all conditions on older individuals. In answer to this it may be said that since it has of late years been positively demonstrated that very young human individuals, even babies, are subject to unmistakable arteriosclerotic lesions under certain conditions, it seems more than probable that the same is true of dogs.

It was now necessary to test the action of acids to better advantage. The acids employed were: (1) hydrochloric acid, to verify if possible the apparently positive result that had been obtained in the two former experiments; (2) lactic acid, because O. Loeb<sup>2</sup> had claimed positive results with this acid and had developed a plausible theory concerning the mechanism of its action; (3) acetic acid, selected because of its occasional occurrence in certain types of digestive disturbance. Other acids, as for instance butyric, might logi-

<sup>2</sup>Loeb, O., Ueber Arteriosklerose. II. Ueber experimentelle Artherienveränderungen mit besonderer Berücksichtigung der Wirkung der Milchsäure auf Grund eigener Versuche, *Deutsch. med. Woch.*, 1913, xxxix, 1819.

cally and profitably have also been tested, but for various reasons this could not very conveniently be done.

### *Hydrochloric Acid.*

The series includes three dogs. All of them are young. Two were males, one female. In every case the ordinary chemically pure hydrochloric acid (specific weight not determined) was used. The initial dose was ten drops mixed with the food of the animal and increased by five drops every 3rd day. In every instance it took some time until the animals became accustomed to this unusual admixture to their diet. They would leave portions of their food, and at times would not eat at all; sometimes the dose of acid would have to be diminished; at other times the administration of the acid would have to be stopped altogether for a few days. But after a longer or shorter period the animals became thoroughly habituated to the acid and ate with great appetite, while the doses of acid were steadily increased. This is true, it should be said, not only of the hydrochloric acid series but of the two other acids as well. In the hydrochloric set there was one experiment (No. 1) that was disappointing.

*Experiment 1.*—Brown dog, male; weight 8,500 gm. Dec. 5, 1914. Commenced with 10 drops increased by 5 drops every 3rd day. Eats well until Dec. 28 by which time the dose reached 65 drops. Now begins to leave some food and to lose weight. Dec. 30. Weight, 7,600 gm. After a few days of 70 drops, dog is kept at 60 from Jan. 1, 1915, to Feb. 10. During all this time, eats well but loses weight steadily. 70 drops up to Feb. 16. Refuses food entirely for 2 days. Feb. 18. Died. Weight at death 5,500 gm. Experiment had lasted only about 2½ months during which time the dog took about 250 cc. of hydrochloric acid.

Besides the usual small punched out erosions in the stomach and the upper intestine and a great number of round worms, there are no definite lesions apparent, except general atrophy and marasmus.

*Experiment 2.*—Young brindle bull, female; weight 9,500 gm. Dec. 1, 1914. Commenced with 10 drops as usual. After a few days, weight begins to decrease. Acid has to be stopped, then again resumed; sometimes the dog eats well, at other times leaves great portions of food. Weight increases slightly, then is again diminished, the lowest weight being 6,800 gm. on Mar. 22, 1915. Appetite, however, is excellent and 80 drops of acid are given daily. From now on the dose of acid is increased by 10 drops about every 2 weeks. Weight increases steadily;



the animal is in excellent condition. Aug. 25. The dog gives birth to six healthy pups. From July 6 to Oct. 19, when the animal was killed, 140 drops were taken daily. Duration of the experiment 10 months, 19 days; total amount of hydrochloric acid consumed somewhat over 2,000 cc.

The autopsy shows a very slight hyperemia in scattered areas through the gastrointestinal tract. Otherwise there is nothing abnormal.

*Experiment 3.*—Black and white dog, male; weight 8,700 gm. Feb. 19, 1915. Dog started at once at 40 drops but though it does not eat well and is losing weight, the dose is steadily increased; weight varies. Though there are times when it absolutely refuses to eat, the dose is still increased and the dog begins to gain steadily in weight. Oct. 15. Killed; weight 9,700 gm. From July 30 to Oct. 15 the dog had taken 140 drops daily, ate all the food, and seemed in excellent health. The experiment lasted about 8 months, during which time the dog had consumed a little more than 1,600 cc.

The autopsy shows besides the usual intestinal parasites, fairly normal conditions. There is not even any very extensive hyperemia in the stomach and intestines.

### *Lactic Acid.*

On the whole it seems that the animals did not take to lactic acid quite so easily as they did to hydrochloric.

*Experiment 4.*—Young fox-terrier, female; weight 6,000 gm. Dec. 1, 1914. Commenced with 10 drops daily; after a week 20 drops were given, and animal refused food, and weight decreased very rapidly. Dec. 10. The lactic acid is stopped altogether. Feb. 16, 1915. Weight is about 4,800 gm. The animal is kept on normal food without any acid until June 4. On that day having been eating well and being in good condition, lactic acid is given again with an initial dose of 90 drops. From now on appetite is excellent and increase in weight steady. The dosage of lactic acid is rapidly increased and from Sept. 19 until Oct. 14, when the dog is killed, 140 drops are taken daily. Weight at death 11,606 gm. The dog was under observation for 10½ months, but the lactic acid experiment, counting from June 4, 1915, lasted a little over 4 months in which somewhat more than 1,000 cc. were administered.

The autopsy shows some patchy hyperemia in the gastrointestinal tract, otherwise no organic lesions. No definite macroscopic signs of arteriosclerosis, although the yellowish ridges and patches in the arch and in the descending aorta are a little more pronounced than usual.

*Experiment 5.*—Bull-terrier, male; weight 13,000 gm. Dec. 1, 1915. Similar history. Eats well for several weeks, though weight decreases. Leaves large portion of food for about a week then begins to eat well again. Weight and appetite vary. Sometimes the dose of lactic acid had to be diminished; sometimes stopped altogether for a day or two. The dose had been increased to  
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175 drops, then it had to be diminished again to 80. Feb. 24, 1916. Lowest weight reached 10,300 gm. From now on dog increases steadily in weight and eats everything. Oct. 8. Killed. From June 14 to Oct. 8, 200 drops of lactic acid taken daily. The experiment lasted about 10 months, the total amount of lactic acid being approximately 3,000 cc.

At the autopsy no gross lesions of any kind are found.

*Experiment 6.*—Brown and white cur, female; weight 10,700 gm. Dec. 29, 1914. History similar to the foregoing. It took until about Feb. 24, 1915, for the animal to become accustomed to the lactic acid. On that day the minimum weight of 6,500 gm. was reached, but from now on the dog showed excellent appetite, ate everything it could get, steadily increasing in weight. Oct. 13. Killed. From June 4 to Oct. 13, 160 drops were taken daily, and the weight at death was 12,700 gm. The experiment lasted about  $9\frac{1}{2}$  months. Total amount of lactic acid consumed somewhat over 2,000 cc. The dog had given birth to a litter of pups 2 days before death.

The autopsy shows no gross lesions.

### *Acetic Acid.*

Again the history is about the same as in the foregoing two series. 50 per cent glacial acetic acid, chemically pure, was used.

*Experiment 7.*—Brindle bull, male; weight 8,100 gm. Dec. 29, 1914. There are the usual variations in weight up to a certain lower limit. The dosage of acid is increased or diminished according to the amount of food the animal takes. It takes till about Mar. 9, 1915, for the dog to become fully accustomed to the acid. From now on its appetite is excellent, health good, and weight steadily increasing. Oct. 7. Killed. From June 14 to Oct. 7 the daily dose was 150 drops. Weight on day of death 12,000 gm. Experiment lasted 9 months and 10 days. Total quantity of glacial acetic acid consumed approximately 2,000 cc.

The autopsy shows no gross lesions.

*Experiment 8.*—Coach dog, male; weight 9,700 gm. Dec. 1, 1914. There are similar variations in weight and in dosage. It takes till Feb. 23, 1915, for the dog to become accustomed to the acid. From then on, dose of acid is steadily increased, and the weight increases though there are occasional bloody stools. June 1. Killed in a fight. Weight at death 10,200 gm. From Apr. 25 to date of death 150 drops of acid were taken daily with excellent appetite. Experiment lasted 6 months. Total amount of glacial acetic acid approximately somewhat over 1,000 cc.

The autopsy shows no gross lesions.

It is not necessary to give any details of the microscopic examinations. No lesion was discovered which could be interpreted as anal-

ogous to arteriosclerosis. The positive findings in the two dogs reported by the writer several years ago were, therefore, evidently not due to the hydrochloric acid, but were spontaneous and probably of the senile type. Loeb's claims of positive results with lactic acid are also not convincing. The one dog 8 years old, Loeb himself disregards on account of his age. In the other dog, only  $1\frac{1}{2}$  years old, he regards as arteriosclerotic various small ridges and patches in the intima of the aorta. He gives no microscopic details and it is therefore not at all unlikely that these patches and ridges were no more significant than those found so frequently in the aorta of normal dogs, both young and old. It can therefore be safely asserted that the feeding of dogs with large quantities of various acids for a comparatively long period, though the cause of weeks of severe indigestion and marasmus, has not resulted in any lesions comparable to arteriosclerosis.

As cholesterol still appears to occupy the most prominent place in studies on arteriosclerosis, it was resolved to revert once more to experiments with that substance. Meanwhile a paper by Klotz<sup>3</sup> had appeared reporting the effects of injections into the ear vein of rabbits of cholesterol dissolved in pure oils or as a soap emulsion. It was decided therefore to try somewhat similar experiments on dogs.

Some attempts were made to develop a technique by means of which cholesterol was to be injected directly into the heart or into the aorta. The only result of these tentative experiments was the production of some cases of hemorrhagic and exudative pericarditis. It was then decided to inject cholesterol through the jugular veins. This series includes four dogs. All four were young animals, probably barely a year old. The cholesterol was obtained from gall stones and thoroughly purified; it was dissolved in sesame oil which was cheap and practically non-toxic. Its phytosterol content, being as in all similar oils less than 1 per cent, could, in the small quantities used in each injection, be considered negligible. It was found that 0.2 cholesterol in about 8 cc. of sesame oil at body temperature gave a perfectly clear solution. This solution was employed exclu-

<sup>3</sup> Klotz, O., Vascular changes following intravenous injections of fat and cholesterol, *J. Med. Research*, 1915-16, xxxiii, 157.

sively. Any attempt to increase the quantity of oil or use a higher concentration of cholesterol gave rise to more or less grave disturbances, as for instance, acute pulmonary edema and rapid death of the animal. For the first few injections, ether was given and a very small incision into the skin was made and the jugular laid bare. It soon became evident, however, that the incision was not necessary and that the vein could be reached with certainty, after a little practice, through the intact skin. Two injections a week were given regularly and without any exception for a period of between 8 and 9 months. The total amount of cholesterol consumed by each animal during that period was about 11 gm. No deleterious effect of the injection was noticed at any time. To observe whether a permanent rise in blood pressure had any effect upon the result of the injections, in two of the dogs, before any injections were made, the abdominal aorta was tied by the Halsted<sup>4</sup> method. A flap of the fascia lata was taken, with which the aorta was tied somewhat below the right renal artery. The ligature was tightened so that pulsation in the femoral artery could just be felt. In both cases the operations were uneventful and the wounds healed without any difficulty.<sup>5</sup> As the autopsies later on showed, permanent stricture of the aorta was accomplished by this operation. This must necessarily have affected not only the pressure in the aortic system, but also, indirectly at least, in the pulmonary circulation into which the cholesterol was primarily discharged.

The further history of these four dogs is uneventful. During the very hot and humid summer of 1916, the animals suffered from the mange, but with the coming of cool weather and with the necessary dermatological attention, they recovered fairly well, and were in good condition when they were killed. One dog did not recover its health entirely after the mange was cured, but continued losing weight. The injections were, however, continued regularly. On November 16 the dog was killed. At the autopsy it was found that the entire intestinal tract, including stomach and colon, showed hemorrhagic

<sup>4</sup> Halsted, W. S., Partial occlusions of the thoracic and abdominal aortas by bands of fresh aorta and of fascia lata, *Proc. Soc. Exp. Biol. and Med.*, 1912-13, x, 113.

<sup>5</sup> The operations were performed by Dr. M. J. Sittenfield, to whom I am indebted for his valuable assistance.

swellings with partial destruction of the muscularis. The contents of the intestines were bloody and the mucosa itself was in many places hemorrhagic. There were numerous ulcers, some of them extending down to the serosa. Great numbers of *Ascaris mystax* were present. In all four dogs the lungs at autopsy were found much firmer than normal and not completely collapsed. There were also present small wedge-shaped hemorrhagic infarctions, and patches of brown pigmentation evidently due to old hemorrhages. The liver, kidneys, and pancreas appeared normal. The adrenals were not enlarged and showed no gross lesions. The spleen in nearly every case was somewhat enlarged and showed very large prominent follicles. In one case there was some softening of the pulp, probably due to hemorrhage. In one of the dogs, in which the aorta had been tied, a definite, fairly close stricture of the vessel was seen surrounded by an area of thickening, but there was no noticeable dilatation proximal to the obstruction. In the other operated dog, there was also an area of thickening about the place of ligature, but the aorta appeared of practically uniform caliber, possibly very slightly constricted at the place where the ligature had been applied. No gross lesion could be recognized in any of the jugulars and the microscopic examination also showed perfectly normal conditions, with only here and there a small droplet of fat in the adventitia. Neither the frequent passage of oil and cholesterol through the vein nor its puncture several hundred times by a rather large needle seems to have had an injurious effect upon the delicate vessel. No trace of inflammation or degeneration could be seen. The heart muscle in every instance was flabby and was found in diastolic dilatation. There was no hypertrophy of the right ventricle. The animals were killed by the injection of chloroform into the jugular vein, and to this the flabby and dilated condition of the heart muscle may be ascribed. The aortas showed some ridges and patches and wrinklins here and there, more frequently around the orifices of branching vessels. These irregularities in the intima of the aorta are usually scarcely visible to the naked eye, especially in the fresh object, unless one has become familiar with them and searches for them carefully. After the artery has been kept in formalin for some time, they become a little more distinct. Besides these, however, more extensive sclerotic thickenings were plainly visible. This was

especially the case in one aorta (Fig. 1) in which, in the sinus Val-salvæ behind the semilunar valves, these sclerotic areas protruded more distinctly. In the bulb of this aorta also and near the orifices of the branching intercostal arteries a number of ridges was noticed which tended to confirm the impression of incipient sclerosis.

The oil and cholesterol injected into the jugular is of course carried through the upper cava and the right auricle into the right ventricle, and from there through the pulmonary artery into the lungs. It might be confidently expected, therefore, that if any changes in the arteries are to result from this procedure, they would show primarily in the pulmonalis, rather than in the aorta, for the first powerful impact of the blood laden with cholesterol is directed against the former. This expectation was completely verified. In all four dogs very definite changes, producing the distinct impression of arterio-sclerotic thickening and loss of elasticity, appeared in the pulmonalis (Fig. 2). There were no signs of ulceration or calcification nor was there any appreciable dilatation. These departures from the normal were principally of the nodular type, protruding more or less prominently into the lumen of the vessel. They commenced immediately behind the valvular cusps, showed the faint whitish yellowish tint common to all sclerotic lesions of the intima, and extended, more or less, further up into the vessel. These nodulations were most pronounced in the two dogs in which the aorta had been tied. In the other two dogs the lesions, though quite definite and plain, were much less in extent and prominence. It is not possible to say whether this is merely coincidental, or whether the increased blood pressure is responsible. The most marked sclerotic changes were found in the dog whose aorta showed comparatively slight obstruction. While the gross lesions of the main trunk of the pulmonary artery closely resembled ordinary intimal arteriosclerosis before the development of atheroma has begun, the microscopic examination revealed a condition entirely unexpected, and in many ways rather surprising. Hypertrophy of the intima with proliferation of the endothelium, new formation of connective tissue, and the splitting of the elastica interna as described by Jores was distinctly in evidence. This hypertrophy of the intima however was comparatively limited and not commensurate with the amount of thickening. In many places there was hardly any prolif-

eration of the intima or it was entirely absent. The most pronounced and constant and evidently primary lesions were found in the media, and especially in its innermost portions adjoining the elastica interna. Within this region there had taken place much damage to the elastic tissue. Everywhere the breaking up of elastic fibers into larger and smaller pieces and into granules (*Körnchenzerfall*) could be seen. Very often it appeared as if the elastic fibers, especially the larger ones, had suddenly snapped and the ends could be seen some distance apart coiled like the lash of a whip. Another lesion, evidently secondary to this forcible disruption of the elastic tissue, was the separation of the muscular elements into bundles of varying dimensions and at varying angles, which were so pushed aside as to leave wider or narrower gaps between them. There seems to be no doubt that this disorganization of the elements of the media was largely responsible for the prominent areas of apparent thickening, and that the proliferation of the intima took only a minor and secondary part in the process (Fig. 3). These comparatively wide gaps between the dislocated groups of muscle cells usually contained remnants of broken up elastic fibers, but otherwise appeared empty (Fig. 4). That they were not empty could be surmised from the compactness and solidity of the nodules. Edema could be positively excluded; staining for fibrin or mucin gave no results, but with certain stains, as for example alum-carmin, the faintest possible tinge could be obtained, sometimes showing slight inequalities and suggesting some very delicate coagulated material. It is probable that these wide areas contain, as Ribbert<sup>6</sup> suggested, coagulated blood plasma. He finds these gaps, however, only in the intima, where he believes they are the effect of excessively high blood pressure. He attributes the deposit of cholesterol to this also. No sign of necrosis could be detected and there was nothing to suggest the median necrosis as seen in rabbits or in human arteries. The muscle cells, although detached from their normal position were in perfect condition, and the elastic fibers though shattered and disorganized retained their normal staining properties. There were no inflammatory

<sup>6</sup> Ribbert, H., Über die Genese der arteriosklerotischen Veränderungen der Intima, *Verhandl. deutsch. path. Ges.*, 1905, viii, 168.

symptoms and the vasa vasorum were normal. It seemed clear that the entire process was purely mechanical. The sclerotic areas in the aorta showed the same median disorganization even more strictly confined to the media, for the intima was mostly not affected, but normal.

The lungs presented a very constant and characteristic microscopic picture. Even a superficial examination showed that the bulk of the oil and its cholesterol content was filtered out through these organs. The sudanophil masses were deposited almost exclusively in the interalveolar septi, and there mainly in the capillaries. The latter were much distended and filled to bursting with oil. Very frequently the distended capillaries were surrounded by an infiltration of leukocytes, both processes together causing a considerable thickening of the septi with very marked encroachment upon the alveolar air spaces. The alveoli themselves were practically free from oil. Here and there an overdistended capillary would burst and a few drops of oil be left in the alveoli. Dispersed throughout the lung there were clear indications of hemorrhages. Numerous red blood corpuscles were found in the air spaces and in the septi, and hemorrhagic infarctions were also irregularly scattered throughout the lungs, especially near the margins. Notwithstanding all this the pulmonary apparatus seemed quite adequate to combat the enormous overburdening caused by the oil injections. Clinically there were no symptoms of respiratory disturbance and this could be accounted for by the fact that though the pulmonary circulation was heavily overtaxed, nevertheless capillaries and alveoli always remained unaffected in sufficient numbers to ensure the necessary aeration of the blood. There is, moreover, good reason to believe that the oil was very rapidly discharged from the lung. If the dog was killed within 24 hours of an oil injection the lungs were found overcrowded with sudanophil masses, while if 2 or 3 days had elapsed between the injection and the killing, comparatively little sudanophil material remained, and the lungs presented an appearance more nearly approaching the normal. It appears that there is a marked difference between the reaction to cholesterol in oil as shown in the lungs of the dog and that described by Klotz<sup>3</sup> in the lungs of the rabbit. A very careful study of a large number of sec-



tions of the lungs of all the dogs and from all parts of the lung failed to show any crystals or any doubly refracting substances. Phagocytes with granular fatty inclusions were scattered throughout the lungs, mostly in the septi, occasionally in the alveoli. Extensive areas of the lung however appeared free from phagocytes. On the other hand, it is the writer's impression that in certain localities, often near the margins, or where the pulmonary tissues had become more or less indurated by hemorrhagic infarctions and the subsequent reactive processes, and where accordingly the circulation was materially interfered with and the blood vessels were presumably no longer able to carry off the fatty material with sufficient dispatch, the phagocytes accumulated in considerable numbers, presumably to replace or rather materially to aid the work of the blood vessels. In such spots large phagocytic cells filled with granular sudanophil material are heaped in close proximity. Frequently several of these cells merge into one large giant cell, the several nuclei being distributed irregularly, some in the center and others pushed aside towards the circumference. Many large cells filled with granules of brown or blackish pigment and resembling very closely the *Herzfehlerzellen* are also found in these indurated areas.

The blood vessels in the dog's lung also showed somewhat different reactions from those described by Klotz for the rabbit. The pulmonary veins as well as the bronchial arteries appeared to be normal. The capillaries also, notwithstanding the tremendous strain to which they were subjected by the overcrowding with oil, did not show structural lesions. The smaller and smallest branches of the pulmonary artery were also without exception normal. It was only in the right and left main branches of the pulmonary trunk and their subdivisions of the second and third order that definite structural changes in the vascular walls were found. It was impossible to decide whether there was any dilatation of these larger vessels as there were no normal measurements available that might have served as a standard, but the general impression was that if any dilatation had actually taken place, it could not be very serious. Several small globular nodules were found in the two main branches, though they were not so large or so extensive as those described in the main trunk. The microscope showed that these little globules were the

result of a very marked and peculiar hyperplasia of the media. The elastic lamellæ and the muscle fibers were not arranged in the usual symmetrical layers but formed more or less regular concentric circles or in other places abutted upon each other at various angles, thus producing upon the whole the impression of a whorl of tissue. Within these peculiar nodulations some of the elastic fibers had also given way and snapped asunder and here also spaces apparently filled with plasma and containing remnants of broken elastic tissue occurred. The dislocation and forcing apart of the elements of the media, and the destruction of elastic tissue are, however, minimal when compared with the same processes as seen in the main trunk. Another peculiarity of this strange form of nodulation is, that it is practically impossible to decide where the media ends or the intima begins. No elastica interna is visible, and the innermost layers of the vessel consist of fibrous and muscular tissue all included in a fine network of delicate elastic fibers forming irregular meshes, and the whole is lined with moderately proliferating endothelium. Rather small phagocytes are occasionally, but on the whole rarely to be found, as also very minute sudanophil droplets between the cells. Very definite and typical hyperplasia of the intima, independent of those hypertrophic median nodules, is also found in the two main branches, but especially in some of the larger arteries well within the lungs (Fig. 5). Here there is comparatively little change in the condition of the media although very small spaces filled with plasma and containing fragments of elastic fibers are sometimes seen just below the elastica interna. These thickenings of the intima, though they distort the lumen of the vessel but slightly, agree accurately with the description given by Jores.<sup>7</sup> There is the splitting of the elastica interna, the new formation of fibrous and muscle cells, and some proliferation of the endothelium, but there is this difference, that there is no pronounced fat phanerosis or fatty degeneration. It is true that some fat phagocytes and minute fatty droplets are found in the intima and even sometimes in the inner layers of the media, but they are few in number and not constant in occurrence or regular in distribution.

<sup>7</sup> Jores, L., *Wesen und Entwicklung der Arteriosklerose*, Wiesbaden, 1903.

It is obvious, therefore, that though the pulmonary circulation was for many months overcrowded with cholesterol in oil; though there must have existed a certain amount of hypercholesteremia, as shown by the excess of sudanophil bodies appearing throughout the heart muscle, the liver, spleen, kidneys, and adrenals; and though the pressure within the pulmonary, and also, though perhaps to a lesser degree in the aortic system, must have been very great; the cholesterol evidently took no essential part in the processes that led to such profound changes in the structure of the arterial walls.

What relations if any, do these findings bear to human atherosclerosis? It is now very generally believed that the latter is an affection primarily of the intima, essentially degenerative in character, and that cholesterol and its esters are very important factors in this process. The results of the present experiments on the other hand are apparently in direct opposition to this theory. Though to the naked eye the nodules protruding into the lumen of the vessels, and also the occasional diffuse thickening of the vascular walls, closely resemble arteriosclerosis at a stage in which ulceration and calcification have not as yet begun, the real localization of the lesion is found to be mainly in the media. The structural disruptions and dislocations appear to be the result of the action of mechanical forces rather than of chemical degenerative reactions. The hypertrophy or hyperplasia of the intima is obviously merely secondary to the lesions in the media and lastly it would appear that cholesterol does not count as a factor in the process. Notwithstanding these seemingly important differences, the present writer is nevertheless of the opinion that a very close analogy, if not identity, exists between human atherosclerosis of the pulmonary blood vessels and the lesions produced experimentally in our dogs. Our knowledge of human atherosclerosis is as yet far from complete. The controversy between those who assume a mechanical disturbance as primarily the cause of arteriosclerosis, those on the other hand who are more inclined to make toxic and other chemical influences responsible, and those who, pursuing a middle course, seek to explain the sclerotic phenomena by combining in various ways the extreme views of both sides, is still unsettled. Aside from the problems of atherosclerosis in general, however, what is known of sclerosis of the pulmonary system must

be taken specially into account. It is not many years ago that it was supposed that sclerosis of the pulmonary artery, if it occurred at all, was extremely rare. Bichat<sup>8</sup> seeks to show that vessels carrying venous blood seldom if ever show traces of sclerosis and hence, according to him, the pulmonary artery, in which only venous blood circulates, is practically exempt. During the last 12 to 15 years the subject has been more intensely studied. A very complete review of the literature on this subject has been given by Posselt,<sup>9</sup> and more recently by Ljungdahl.<sup>10</sup>

It is fairly certain now that pulmonary sclerosis is found quite frequently if it is only carefully looked for. Genuine senile sclerosis of the pulmonary artery seems to be quite rare, but according to Ljungdahl, contrary to the view of many authors, it does occur, but mainly in association with special complications, pulmonary emphysema for instance, that tend to raise the pressure within the artery considerably. Fischer<sup>11</sup> found in several cases of advanced atherosclerosis of the aorta and peripheral arteries, that the pulmonary arteries were healthy, but in these cases the lungs were free from emphysema or other chronic lesions and there was no hypertrophy of the right heart. Posselt finds that sclerosis of the pulmonary artery seems to affect youthful persons more readily than is the rule in sclerosis of the aortic vessels. According to this author 97 out of 187 cases were found in individuals under 40, 125 in subjects under 50, and only 60 in persons above 50 years of age. All authors, however, seem agreed that hypertension within the pulmonary circulation is a main factor in the etiology of sclerosis of the pulmonary artery, which is generally looked upon as an *Abnutzungskrankheit* as defined by Marchand.<sup>12</sup> The fact frequently observed that the artery remains intact, though the blood pressure is steadily much above normal, strongly suggests that hypertension is not the only factor, but that other etiological elements, such as local or functional variations, toxic or other influences as yet unknown, are also involved. For the pulmonary artery too, the dogma that the intima is the primary seat of the pathological changes is very generally accepted.

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<sup>8</sup> Bichat, quoted by Brüning, H., Untersuchungen über das Vorkommen der Angiosklerose im Lungenkreislauf, *Beitr. path. Anat. u. allg. Path.*, 1901, xxx, 457.

<sup>9</sup> Posselt, A., Die Erkrankungen der Lungenschlagader, *Ergebn. allg. Path. u. path. Anat., 1te Abt.*, 1909, xiii, 298.

<sup>10</sup> Ljungdahl, M., Untersuchungen über die Arteriosklerose des kleinen Kreislaufs, Wiesbaden, 1915.

<sup>11</sup> Fischer, W., Über die Sklerose der Lungenarterien und ihre Entstehung, *Deutsch. Arch. klin. Med.*, 1909, xcvi, 230.

<sup>12</sup> Marchand, *Verhandl. Kong. inn. Med.*, 1904, xxi, 23.

There are, however, some dissenting voices. Ehlers<sup>13</sup> finds that the human pulmonary artery differs from the aorta in that its media is made up of two distinct layers and according to him the innermost layer assumes a very active part in pathological processes. Durante<sup>14</sup> reports a case of congenital atheroma in a premature infant. He finds the vasa vasorum normal, the intima almost normal. The main process is in the inner third of the media where there is some necrosis and calcification. He also describes broad, almost unstainable gaps in the media. There is some secondary hypertrophy of the intima but only over points of most advanced degeneration of the media. The aorta appears macroscopically normal, but under the microscope it is seen that in its media there are similar processes developing as in the pulmonary. Except for the median necrosis and calcification, the conditions in this case seem to bear a striking resemblance to those obtained experimentally in our dogs.

These few brief notes from the literature will suffice to show that the results of the experiments here described present some obvious analogies to the sclerosis of the human pulmonary artery, but at the same time distinct differences. The proliferation of the intima wherever it is found is the exact counterpart of what is considered typical for human atherosclerosis, and in the section taken from a branch within the lung in which the media is barely affected (Fig. 5) the picture is identical with any that can be taken from an arteriosclerotic artery that has not as yet undergone an appreciable amount of degeneration. One of the chief points of difference is that in our dogs it is not the intima but the media which is without doubt the primary and principal localization of the lesion. That this is not necessarily peculiar to the dog but can be found also in human pulmonary arteries is sufficiently proved by the above quotations from Ehlers and Durante. Perhaps greater experience and improved methods may bring forward a greater number of similar human cases. Our experiments, moreover, have shown the process at a much earlier stage than is usually seen in the human. That the peculiar structure of the pulmonary artery as described by Ehlers is not to be held responsible for this localization in the media is obvious because identical lesions may be found at the same time in the aorta.

<sup>13</sup> Ehlers, W. E., *Zur Histologie der Arteriosklerose der Pulmonalarterie*, *Virchows Arch. path. Anat.*, 1904, clxxviii, 427.

<sup>14</sup> Durante, G., *Athérome congénital de l'aorte et de l'artère pulmonaire*, *Bull. Soc. anat. Paris*, 1899, lxxiv, 97.

One is apt, therefore, to recur to the original theory of Thoma<sup>15</sup> who assumed some defect in structure or in function of the media as the initial stage in the development of atherosclerosis. The almost uncomplicated hyperplasia of the intima as seen in some of the larger arteries within the lung itself might also be readily accounted for by the early theories of Thoma. These arteries are without doubt dilating under pressure, the current is probably considerably slower than normal, and hence the compensatory thickening of the intima. It might be assumed that the effect upon the media was comparatively slight because in arteries of that order the muscular apparatus is not sufficiently developed to exert any great resistance to the pressure within the artery. The disorganization in varying degree of elastic lamellæ and fibers is admitted by all observers but is generally conceived as taking place principally in the intima, and only to a very limited extent in the media. It is taken to be a direct consequence of fatty degeneration. According to Torhorst<sup>16</sup> degeneration does not commence before the completion of the hyperplastic process in the intima, and he as well as most other investigators, especially Aschoff,<sup>17</sup> see the cause of the breaking up of the elastic elements in the fatty degeneration of the cement (*Kittsubstanz*) that serves to hold the fibers together.

In the present set of experiments, no trace of degeneration, fatty or otherwise, could be found. The violent destruction of the elastic elements, the forcible displacement of the muscle cells, the broad gaps filled with plasma, all point to a purely mechanical force, in all probability the intense straining of the musculature of the arteries to overcome the resistance offered by the extensive blocking of the pulmonary circulation in the lung. It will be of great interest to observe whether the cholesterol injections, possibly with some modifications, and continued during a much longer period, will eventually lead to fatty degeneration and calcification, though Ljungdahl seems to believe that 7 to 8 months of increased resistance on the part of

<sup>15</sup> Thoma, R., Über die Histomechanik des Gefäßsystems und die Pathogenese der Angiosklerose, *Virchows Arch. path. Anat.*, 1911, cciv, 1.

<sup>16</sup> Torhorst, H., Die histologischen Veränderungen bei der Sklerose der Pulmonalarterie, *Beitr. path. Anat. u. allg. Path.*, 1904, xxxvi, 210.

<sup>17</sup> Aschoff, L., Arteriosklerose, *Beihft med. Klin.*, 1914, x, 1.

the lungs and increased pressure from the right heart is a sufficiently long time to permit degenerative changes to develop in human pulmonary arteries.

One would think that if cholesterol or its esters participate actively in causing sclerotic lesions, such activity would be plainly demonstrated by the present experiments. That cholesterol is practically inert, that no esters, at least no doubly refracting bodies, have been met with, appears to be one of the striking differences between the arterial lesions of our dogs and human atherosclerosis. This discrepancy is, however, not so great as it would seem. The modern trend of opinion, especially as represented by Aschoff, seems to be that deposits of cholesterol esters are secondary to the mechanical conditions by means of which the intima, and perhaps also the media, undergoes serious structural alterations. Plasma is forced into the intima and the amount of lipid deposit that takes place depends largely on the cholesterol ester content of that plasma. With this precipitation of lipoids, degeneration begins, the *Kittsubstanz* is demolished, and those processes are initiated that lead to atheroma.

This conception is, however, not as yet a well grounded fact, but rather an hypothesis. It is true that cholesterol and its esters are invariably found in every case of human atheroma and that the extent of the degeneration is approximately proportionate to the quantity of lipoids accumulated within the vascular tissues, but up to date nothing definite is as yet known about the exact conditions that induce the lipid deposits or of the specific functions of the latter. Perhaps there may be some truth in the theories propounded by Lemoine and Gèrard,<sup>18</sup> according to which the cholesterol acts as an antitoxin and is heaped up in the degenerating vascular tissues in order to neutralize and to assist in transporting the toxic material injected into the blood vessels by the various processes and diseases that are supposed to lead to arteriosclerosis. In this connection it is significant that Klotz<sup>3</sup> finds that the hemolytic properties of sodium oleate are neutralized by an adequate admixture of cholesterol. Some added interest as regards the function of cholesterol in arterio-

<sup>18</sup> Lemoine and Gèrard, in Bertrand, *Nature de l'athérome et rôle de la cholestérine dans sa formation*, *Rev. mod. méd. et Chir.*, 1913, xi, 380.

sclerosis may also be found in recent studies of sclerotic lesions as found in very young persons and babies, especially by Stumpf<sup>19</sup> and Saltykow.<sup>20</sup> The present writer has also examined a relatively large number of infantile aortas, of several fetuses, and also of dogs. He hopes to publish his results in the near future, but it may be stated now that in the majority of the baby aortas, no matter what the cause of death, no matter how old or how young the child, minute ridges and patches slightly elevated above the level of the intima, and of a faint yellowish color, could be found. They were most frequently found in the sinus Valsalvæ, the bulb or the arch, but often in the descending and abdominal aorta and usually in the neighborhood of the orifices of branching vessels. Under the microscope there could regularly be seen a very distinct and typical hyperplasia of the intima, a practically intact media, very minute sudanophil droplets scattered in the interstices between the elements of the intima, and also fairly large phagocytes filled with sudanophil granules. The same phenomena could with great constancy be found also in the aorta of young dogs. In these cases there were no signs of degeneration, nor did there appear to be any tendency towards atheroma, and it must be a subject for further study to ascertain the functions of the cholesterol, and for what reason and by what mechanism a baby 1 day old or a 7 months fetus had small areas of hyperplastic intima and sudanophil phagocytes in the aorta. It seems probable that the distribution and the presumptive action of cholesterol in these cases and that found in the pulmonary arteries of our dogs are similar.

As a result of this brief discussion it is believed that the arterial lesions experimentally obtained in our dogs are essentially identical with those found in the human pulmonary artery, and that for the first time atherosclerosis, very closely analogous to the human, has with certainty been produced experimentally in dogs.

<sup>19</sup> Stumpf, R., Über die Entartungsvorgänge in der Aorta des Kindes und ihre Beziehungen zur Atherosklerose, *Beitr. path. Anat. u. allg. Path.*, 1914, lix, 390.

<sup>20</sup> Saltykow, S., Jungendliche und beginnende Atherosklerose, *Cor.-Bl. Schweiz. Ärzte*, 1915, xlv, 1057, 1089.



## SUMMARY.

Cholesterol dissolved in sesame oil and injected regularly for a period of from 7 to 8 months into the jugular vein of four young dogs has caused in each animal larger and smaller nodules protruding to some degree into the lumen of the pulmonary artery and also here and there some diffuse thickenings, the whole closely resembling human arteriosclerosis. These changes commence at the origin of the pulmonary artery immediately behind the semilunar valves. They seem a little more pronounced in those dogs that had the aorta tied. Microscopically the lesions are seen to be primary in the media. They consist of a more or less violent disorganization of the elastic elements and displacement of the muscular tissue. As a consequence wide gaps, apparently filled with plasma, are formed in the media. The entire process is localized principally in the inner third of the media. There is no evidence of fatty degeneration either in the elastic elements or the muscle. Typical sclerotic hyperplasia of the intima is found over some of the prominent median nodulations while over others the intima is normal. Hyperplastic sclerosis of the intima is also found in places where the media is intact or only slightly damaged. Some of the larger branches of the pulmonary artery well within the lung tissue are somewhat dilated and show extensive hyperplasia of the intima over almost normal media. Peculiar small nodules are seen in the media of the two main branches of the pulmonary artery which are found to consist of frank hyperplasia of the elastic and muscular elements arranged in more or less regular concentric circles. The capillaries and smaller and smallest arteries within the lungs are not affected. Cholesterol in phagocytes and in droplets between the tissue elements is present in very small quantities and does not take any active part in the process. The entire process represents in all probability an early stage of arteriosclerosis, a result of purely mechanical stress brought about by the very extensive but more or less intermittent blocking of the pulmonary circulation by the oil. The musculature of the artery appears to be the main force applied to overcome the resistance in the circulation. In no case has hypertrophy of the right heart been found.

The lesions in the pulmonary arteries of the dogs produced experimentally are closely analogous to atherosclerosis of the human pulmonary artery.

#### EXPLANATION OF PLATES.

##### PLATE 40.

FIG. 1. Left heart and aorta of a dog whose aorta had been tied, showing sclerotic nodules behind the cusps and ridges in the further course of the vessel.

FIG. 2. Extensive sclerosis of the pulmonary artery, possibly some dilatation of the right ventricle.

##### PLATE 41.

FIG. 3. The widespread disorganization of the media, the large gaps containing fragments of elastic fibers, and faintly stained coagulated material are shown. Muscle cells normal, extensive splitting of elastica interna. No change in the intima. Hematoxylin and Sudan III; Zeiss oc. 4, apochromatic 3 mm.

##### PLATE 42.

FIG. 4. Frozen section. The disruption of the inner portions of the media and moderate hyperplasia of the intima are shown. The elastica interna is only partially intact. Hematoxylin and Sudan III; Zeiss oc. 4, apochromatic 8 mm.

##### PLATE 43.

FIG. 5. Paraffin section of a branch of the pulmonary artery within the lung. Typical sclerotic hyperplasia of the intima, some disorganization of the inner portions of the media. To the right of the picture the intima with its endothelium is seen to be perfectly normal. Hematoxylin and eosin; Zeiss oc. 4, apochromatic 16 mm.

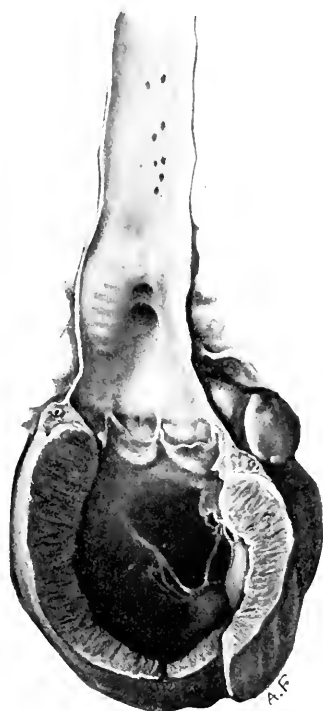


FIG. 1.

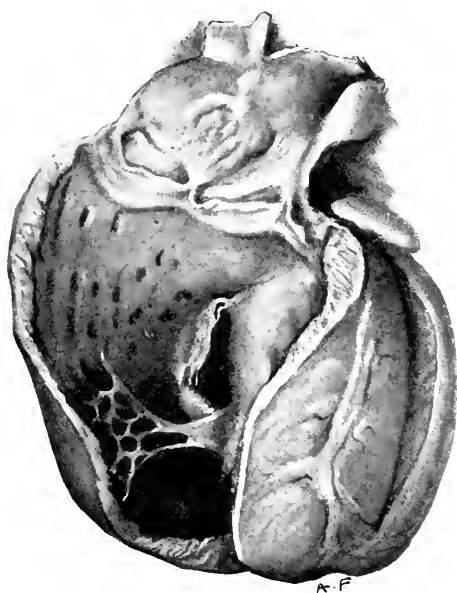
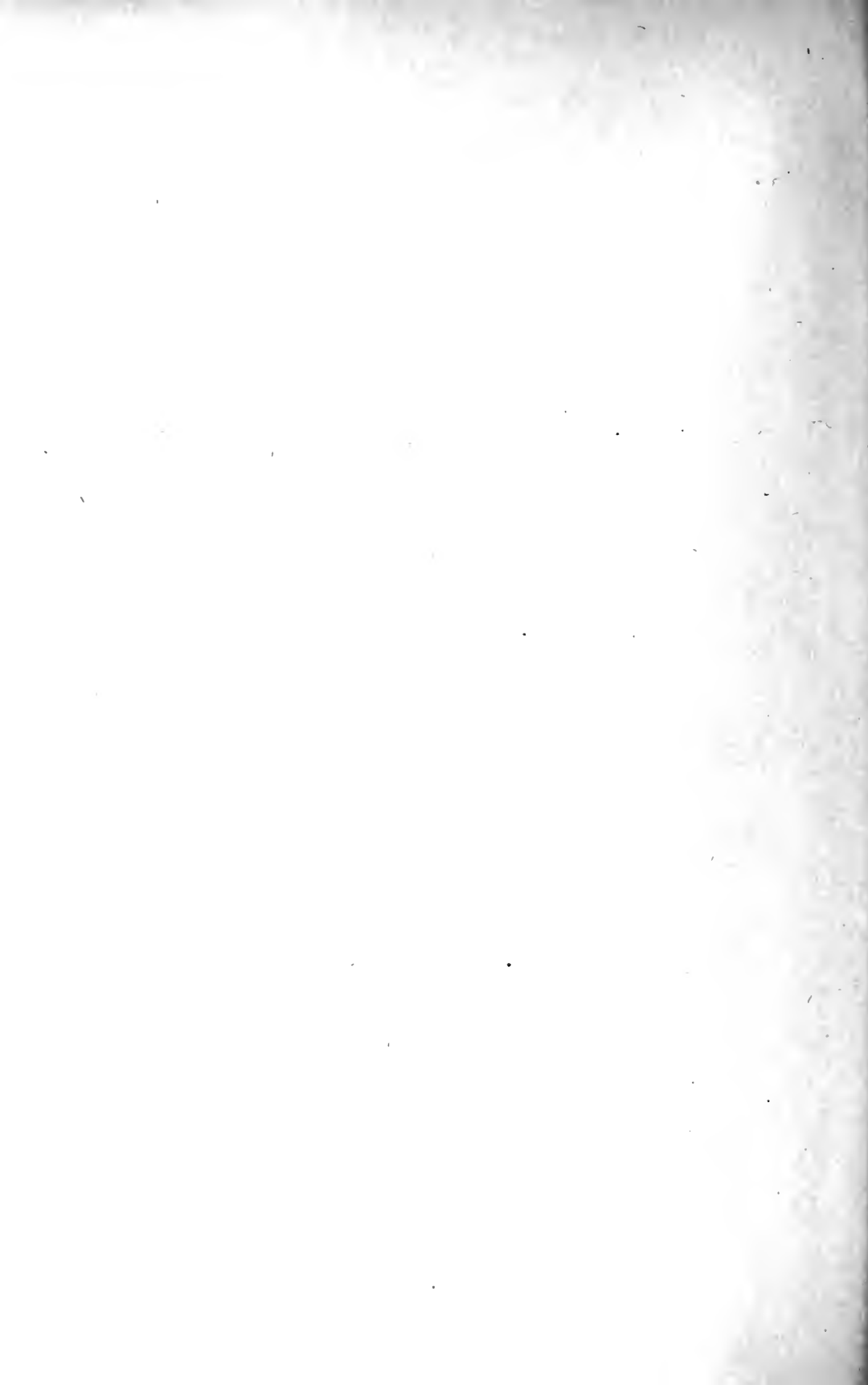


FIG. 2.

(Adler: Experimental atherosclerosis.)



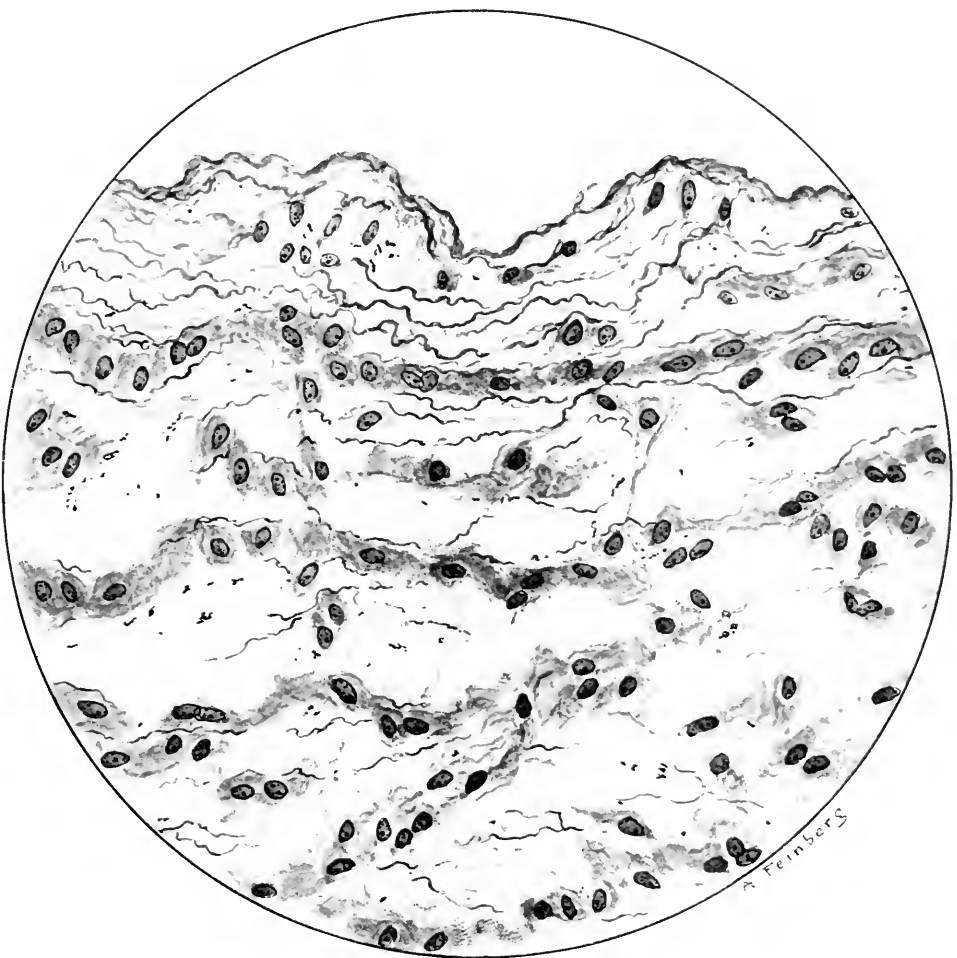


FIG. 3.

(Adler: Experimental atherosclerosis.)

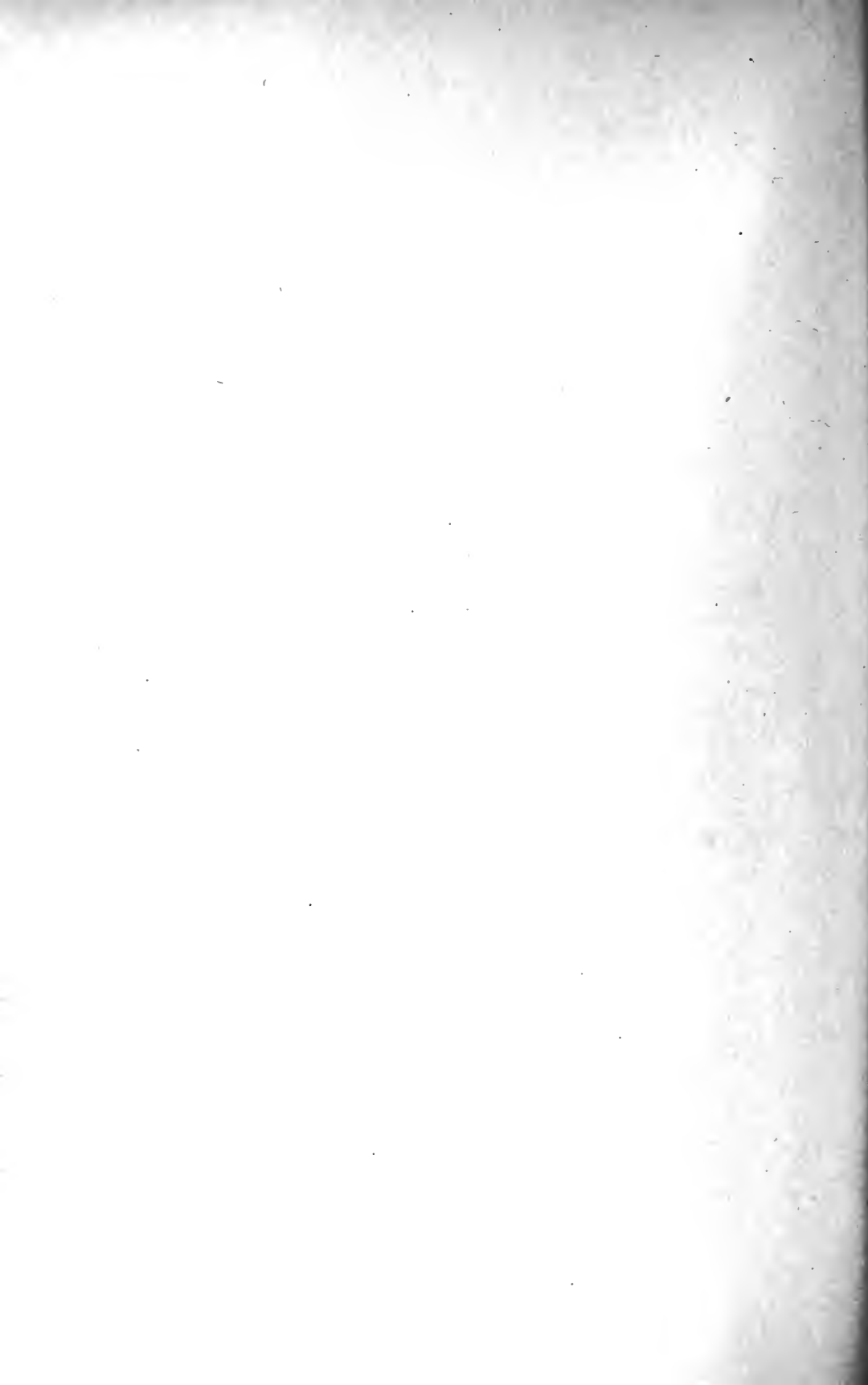
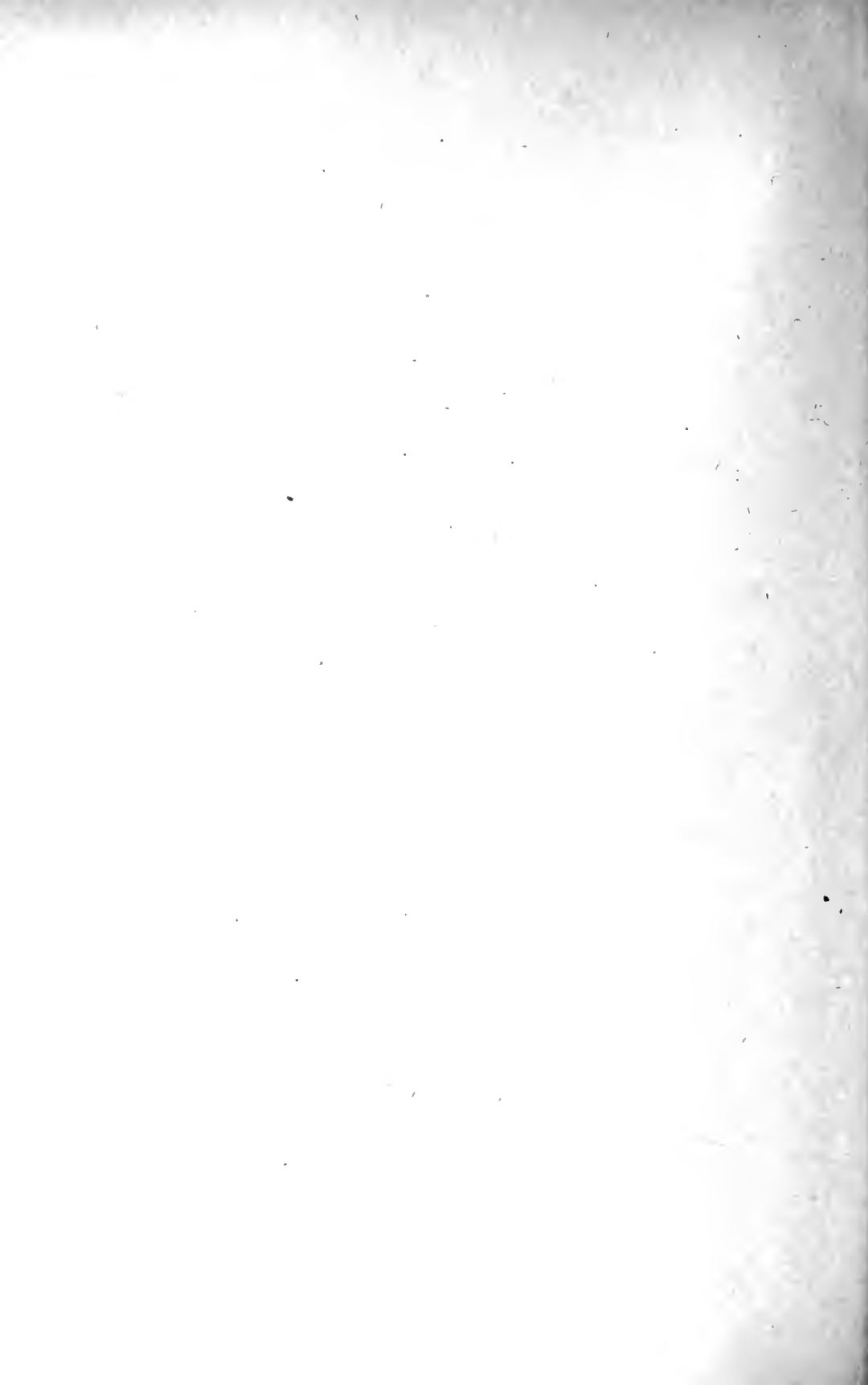




FIG. 4.

(Adler: Experimental atherosclerosis.)





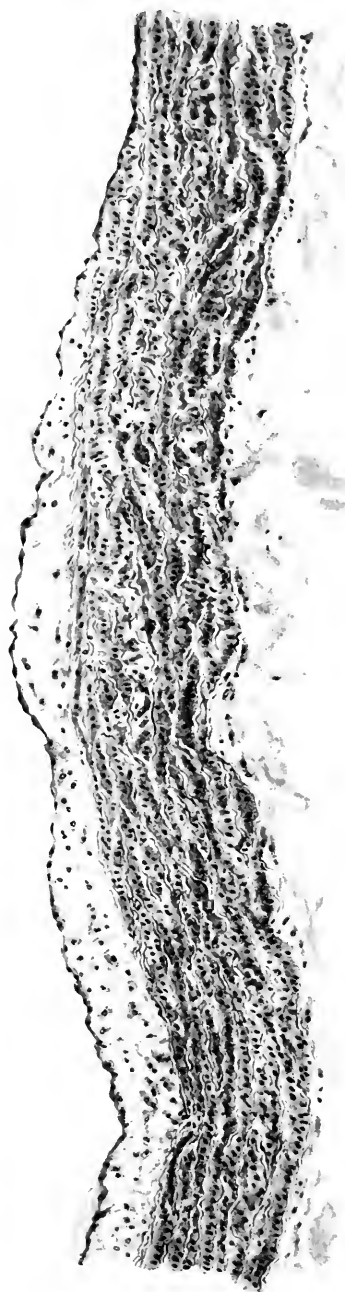
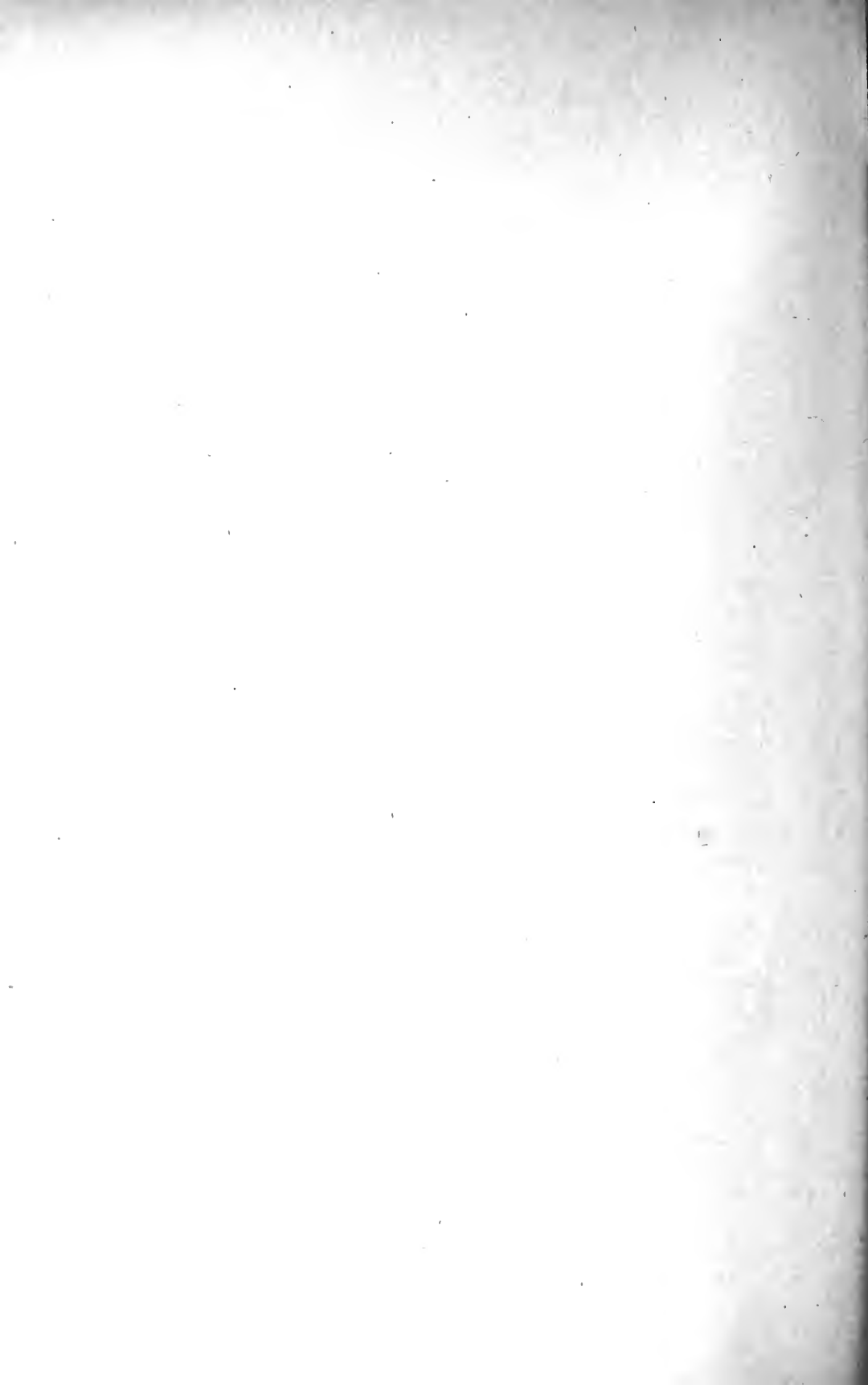


FIG. 5.

(Adler: Experimental atherosclerosis.)



# THE PROPHYLACTIC AND THERAPEUTIC PROPERTIES OF THE ANTITOXIN FOR *BACILLUS WELCHII*.

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(Received for publication, June 1, 1917.)

In a recent article<sup>1</sup> experiments were given which seemed to establish the following points: (1) under certain defined conditions, a bacteria-free toxic substance can be obtained from cultures of *Bacillus welchii*; (2) this substance possesses the physical properties of an exotoxin and, on animal inoculation, is capable of producing all the essential lesions and effects of infection with the bacilli; (3) animals which have received a number of graded doses of the toxin yield an immune serum which neutralizes *in vitro* all the pathologic effects of the toxin and exhibits power to prevent and control infections with both the spore and vegetative forms of the bacilli. These experimental results appeared to justify the conclusion that *Bacillus welchii* should be classed with *Bacillus diphtheriae* and *Bacillus tetani* as a toxin-producing organism and that infections with the organism might be successfully combated by means of a specific immune serum. The present paper deals with more extensive and systematic experiments on the preventive and curative powers of the antitoxin.

## *Prophylaxis against Intoxication.*

A series of guinea pigs ranging in weight from 500 to 800 gm. was given a prophylactic dose of antitoxic serum. Each animal received 0.25 cc. of the antitoxin<sup>2</sup> for each 100 gm. of weight, and the susceptibility of the prophylactic series to intravenous injections of the toxin

<sup>1</sup> Bull, C. G., and Pritchett, I. W., *J. Exp. Med.*, 1917, xxvi, 119.

<sup>2</sup> This antitoxin has now been prepared in the horse. The method employed for producing it will be the subject of a later paper, as well as the question of standardization. At present dosage is given in numbers of cubic centimeters of the serum employed.

was compared with that of normal guinea pigs at different intervals after the immune serum had been given. The following protocols give the results:

*Experiment 1. 2 Days after the Prophylactic Dose of Antitoxin.*—Guinea Pig 1, weight 510 gm., had received 1.5 cc. of the antitoxin subcutaneously. 10 cc. of toxin were injected into the jugular vein. No symptoms had developed 6 hours after the injection, but the guinea pig was found dead 20 hours after receiving the toxin. The autopsy showed lesions characteristic of *B. welchii* toxin.<sup>3</sup>

Guinea Pig 2, weight 660 gm., had received 1.6 cc. of antitoxin subcutaneously. 8 cc. of toxin were injected into the jugular vein. The guinea pig developed no symptoms and was discarded in perfect condition 3 weeks later.

Guinea Pig 3, weight 560 gm., normal control. 0.3 cc. of toxin was injected into the jugular vein. This guinea pig was found dead 16 hours later. Characteristic lesions were found at autopsy.

*Experiment 2. 5 Days after the Prophylactic Dose of Antitoxin.*—Guinea Pig 4, weight 660 gm., had received 1.6 cc. of antitoxin subcutaneously. 4 cc. of toxin were injected into the jugular vein. This animal developed symptoms of intoxication 2 hours after the injection and died at the expiration of 4 hours. The autopsy findings were characteristic.

Guinea Pig 5, weight 510 gm., had received 1.5 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No immediate or delayed symptoms arose, and the guinea pig was in perfect health 3 weeks later.

Guinea Pig 6, weight 770 gm., normal control. 0.25 cc. of toxin was injected into the jugular vein. The guinea pig was found dead 16 hours later. The autopsy findings were typical.

*Experiment 3. 7 Days after the Prophylactic Dose of Antitoxin.*—Guinea Pig 7, weight 670 gm., had received 1.7 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No symptoms developed, and the guinea pig was normal 3 weeks later.

Guinea Pig 8, weight 600 gm., normal control. 1 cc. of toxin was injected into the jugular vein. The guinea pig died 3½ hours later, and characteristic lesions were present.

*Experiment 4. 9 Days after the Prophylactic Dose of Antitoxin.*—Guinea Pig 9, weight 650 gm., had received 1.6 cc. of antitoxin subcutaneously. 2 cc. of toxin were given intravenously. No symptoms had appeared 6 hours later, but the guinea pig was found dead 24 hours after the toxin was given. The autopsy findings were characteristic of *B. welchii* intoxication.

Guinea Pig 10, weight 650 gm., had received 1.6 cc. of antitoxin subcutaneously. 1.5 cc. of toxin were injected into the jugular vein. No symptoms of intoxication arose, and the guinea pig remained normal.

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<sup>3</sup> Differences in the manner of action of *B. welchii* toxin when administered by intravenous and subcutaneous routes are described in the paper already referred to.<sup>1</sup>

Guinea Pig 11, weight 700 gm., normal control. 0.7 cc. of toxin was given intravenously. Death occurred 4 hours later, and the lesions were typical.

*Experiment 5. 12 Days after the Prophylactic Dose of Antitoxin.*—Guinea Pig 12, weight 640 gm., had received 1.6 cc. of antitoxin subcutaneously. 0.7 cc. of toxin was injected into the jugular vein. No symptoms had arisen 8 hours later. The guinea pig was found dead 22 hours after the toxin was given, and the autopsy findings were typical.

Guinea Pig 13, weight 660 gm., had received 1.7 cc. of antitoxin subcutaneously. 0.5 cc. of toxin was injected into the jugular vein. The guinea pig remained normal.

Guinea Pig 14, weight 600 gm., normal control. 0.28 cc. of toxin was given intravenously. This guinea pig was found dead 20 hours later. The autopsy findings were typical.

*Experiment 6. 14 Days after the Prophylactic Dose of Antitoxin.*—Guinea Pig 15, weight 570 gm., had received 1.5 cc. of antitoxin subcutaneously. 0.25 cc. of toxin was injected into the jugular vein. Death occurred 20 hours later. Characteristic toxin lesions were found at autopsy.

Guinea Pig 16, weight 580 gm., had received 1.5 cc. of antitoxin subcutaneously. 0.25 cc. of toxin was given intravenously. No symptoms developed, and the guinea pig was in perfect health 3 weeks later.

Guinea Pig 17, weight 600 gm., normal control. 0.27 cc. of toxin was injected into the jugular vein. The animal was found dead 20 hours after the toxin was given, and typical lesions were present.

The foregoing experiments show that a passive immunity to *Bacillus welchii* toxin can be conferred on guinea pigs by the administration of the antitoxin. In these instances the immunity persisted for about 2 weeks. The protection from the toxin was at first very pronounced, thirty-two acutely lethal doses being harmless when injected directly into the blood stream (Experiment 1, Guinea Pig 2) 2 days after the antitoxin was given. 5 days after the prophylactic administration of the antitoxin, eight lethal doses were still harmless (Experiment 2, Guinea Pig 5), but sixteen lethal doses killed. The immunity to the toxin gradually decreased and was about exhausted at the end of 2 weeks. At this time some of the prophylactic animals were still somewhat more resistant to the toxin than normal animals, while others manifested no increased resistance (Experiment 6, Guinea Pigs 15, 16, and 17).

The fact that animals can be passively immunized to the toxin may in itself be of considerable practical significance; but when it is remembered that the toxin is a powerful aggressin, preparing the field

for bacterial multiplication, its high significance becomes at once apparent. The next series of experiments further emphasizes the importance of the part played by the toxin in infection by the bacilli and shows conclusively that large numbers of highly virulent bacilli are practically harmless when deprived of their toxin.

*Prophylaxis against Infection.*

A series of guinea pigs, ranging in weight from 250 to 350 gm., was given subcutaneously on the inner aspect of one hind leg 1 cc. of antitoxin per 100 gm. of weight. The resistance of the prophylactic series to infection with fresh cultures of the virulent bacilli was compared with that of normal guinea pigs of the same size. The protected guinea pigs were infected subcutaneously in the leg opposite to the one in which they had received the antitoxin. The quantity of culture given the individual animals was calculated on the basis of body weight, a certain fraction of a cubic centimeter being given for each 100 gm. of weight. The following protocols illustrate the results:

*Experiment 7. 24 Hours after the Prophylactic Dose of Antitoxin.*—Guinea Pig 18, weight 280 gm., had received 2.8 cc. of antitoxin. 0.28 cc. of culture was given subcutaneously. 7 hours later the infected leg was moderately swollen and stiff; no local crepitation and no symptoms of general intoxication. 24 hours after the inoculation the local swelling had almost disappeared, and the guinea pig was apparently well.

Guinea Pig 19, weight 280 gm., had received 2.8 cc. of antitoxin and was given 0.5 cc. of culture. 7 hours later the infected leg was swollen and stiff; crepitation could not be elicited, and there was no general intoxication. The next day the swelling and stiffness were subsiding, the guinea pig was active, and no extension of the infection arose.

Guinea Pig 20, weight 250 gm., had received 2.5 cc. of antitoxin and was given 0.9 cc. of culture. The results were identical with those of the two preceding animals.

Guinea Pig 21, weight 310 gm., normal control. 0.006 cc. of culture was given subcutaneously. This guinea pig was found dead 22 hours after it was inoculated. Autopsy revealed edema, gas, and disorganization of tissue, lesions typical of *B. welchii* infection.

In Experiment 7 it is shown that 50, 83, and 150 lethal doses of culture respectively failed to infect the guinea pigs which had re-

ceived a protective injection of antitoxin 24 hours previously. As 150 lethal doses was the largest quantity of culture given the height of the resistance to infection was not accurately determined.

*Experiment 8. 3 Days after the Prophylactic Dose of Antitoxin.*—Guinea Pig 22, weight 250 gm., had received 2.5 cc. of antitoxin and was given 0.4 cc. of culture. No symptoms developed aside from local swelling and stiffness which rapidly subsided. No evidence of multiplication of the bacilli.

Guinea Pig 23, weight 310 gm., had received 3.1 cc. of antitoxin. 0.6 cc. of culture was injected into the opposite leg. The symptoms and results were the same as in the preceding animal.

Guinea Pig 24, weight 300 gm., normal control. 0.004 cc. of culture was given subcutaneously. The animal died 22 hours later. Lesions of *B. welchii* infection were present at autopsy.

In this experiment the limit of the resistance to infection was again not reached, although one of the guinea pigs also received 150 lethal doses of the culture.

*Experiment 9. 5 Days after the Antitoxin.*—Guinea Pig 25, weight 260 gm., had received 2.6 cc. of antitoxin. 1.5 cc. of culture were injected into the opposite leg. Infection did not develop, and the local swelling and stiffness which always follow these inoculations rapidly subsided.

Guinea Pig 26, weight 280 gm., had received 2.8 cc. of antitoxin. 2 cc. of culture were given. Infection developed, and the guinea pig died 30 hours after inoculation. Lesions typical of *B. welchii* infection were present at autopsy.

Guinea Pig 27, weight 290 gm., normal control. 0.005 cc. of culture was injected subcutaneously. The guinea pig was found dead 20 hours later and showed typical lesions.

This experiment indicates that a protected guinea pig could resist 300 but not 400 lethal doses of the culture 5 days after the antitoxin had been given.

*Experiment 10. 8 Days after the Antitoxin.*—Guinea Pig 28, weight 260 gm., had received 2.6 cc. of antitoxin. 0.3 cc. of culture was given subcutaneously. The usual local swelling and stiffness followed, but no infection arose.

Guinea Pig 29, weight 280 gm., had received 2.8 cc. of antitoxin subcutaneously. 0.5 cc. of culture caused a fatal infection, with characteristic lesions.

Guinea Pig 30, weight 275 gm., normal control. 0.005 cc. of culture was given subcutaneously. Infection developed, and the guinea pig died 23 hours after inoculation.

This experiment shows that 60 lethal doses of culture failed to infect 8 days after the antitoxin had been administered. The resistance of the protected guinea pigs was tested again on the 11th day, and twenty lethal doses were not infectious. On the 14th and 15th days, however, the antitoxin-treated guinea pigs proved susceptible to infection, but whether to the same degree as the normal controls the experiment does not indicate. We may, however, assume that the antitoxin becomes greatly reduced in quantity from the 12th to 13th day after its administration.

#### *Therapeutic Property of the Antitoxin.*

We may now consider the effects which the antitoxin exerts upon established infection with *Bacillus welchii*. For this purpose guinea pigs weighing from 500 to 600 gm. were used, smaller ones being so susceptible to the infection that there is little time afforded for treatment. The amount of a standard culture that would infect and kill all of a series of guinea pigs between 30 and 48 hours was first determined. The infecting dose was calculated on the basis of body weight, and it was found that about 0.035 cc. of culture per 100 gm. of weight was effective within the time limit mentioned in all the animals inoculated ranging in weight from 500 to 600 gm. The procedure was to infect a number of animals at one time with the same culture and allow 24 hours to elapse before beginning treatment. At this time the condition of the animals was noted, especially the extent of the local lesions and the symptoms of general intoxication. One of the animals was etherized and autopsied, and to one or more the antitoxin was administered, while the others were left as untreated controls. Illustrative protocols follow:

*Experiment 11.*—Guinea Pig 31, weight 510 gm. 0.18 cc. of culture was injected into the muscles on the inner aspect of the hind leg. 24 hours after inoculation, a gaseous phlegmon involving the infected leg and the adjacent abdominal wall had developed, crepitation was readily elicited, and the guinea pig exhibited symptoms of severe intoxication—rough coat, rapid respiration, and drowsiness. 3 cc. of antitoxin were injected into the jugular vein. The next day the local lesion was subsiding, crepitation could not be elicited, the toxic symptoms had disappeared, and the guinea pig ate greedily. 3 cc. of antitoxin were given sub-



cutaneously to prevent a recrudescence of the infection. The general condition improved from day to day, while the infected leg became gangrenous, sloughed, and was infected with pyogenic organisms. Healing finally occurred, but the scar tissue caused deformity of the leg.

Guinea Pig 32, weight 560 gm. 0.2 cc. of culture was injected into the muscle on the inner aspect of the hind leg. 24 hours after inoculation there were swelling and edema, crepitation, and intoxication. The animal was not treated, and it died exactly 48 hours after inoculation. Autopsy revealed the characteristic lesions of *B. welchii* infection.

Guinea Pig 33, weight 580 gm. 0.2 cc. of culture was injected in the same manner as in Guinea Pigs 31 and 32. 24 hours after inoculation, the guinea pig was etherized and autopsied. There was a large gaseous phlegmon involving the infected leg and adjacent abdominal wall. The muscles of this region were pulplified and laden with bacilli. The opposite groin contained a gelatinous, serosanguineous exudate. The muscles of the abdominal wall and diaphragm were of pinkish hue. The lungs were pink and edematous.

Guinea Pig 34, weight 605 gm., was inoculated with 0.21 cc. of culture. This animal served as an untreated control and died at the end of 40 hours of typical *B. welchii* infection.

Experiment 11 was repeated a number of times, and it was found that the infection could be regularly arrested by the antitoxin after it was well established and extensive destruction of the tissues in the region of the infection had already taken place. Symptoms of severe general intoxication were always present at this stage of the disease. The effect of the antitoxin was often noticeable within from 30 minutes to 1 hour after its administration. The treated guinea pigs would become more active, their coats smooth, and they would be attracted by food, while the controls remained crouched in a corner, with heads down and coats rough, and could not be induced to eat.

#### DISCUSSION.

The experimental results here reported with the preventive and therapeutic applications of the antitoxin are highly suggestive. They derive significance from the fact that *Bacillus welchii* infections in guinea pigs and other susceptible animals<sup>4</sup> are comparable with in-

<sup>4</sup> Protection and curative experiments have been carried out also with the more highly susceptible pigeon. They confirm the results with guinea pigs with certain variations due to the difference in species.

fections with this organism in man. The experimental infections in the guinea pig differ, however, from the natural infection in man in two important points: (1) man possesses a higher natural resistance to infection; (2) the guinea pigs were infected with fresh virulent cultures, while man must, in the great majority of instances, derive infection from spores. Moreover, the protected guinea pigs were given many lethal doses of the living cultures. Such massive inoculations do not occur in man. It may therefore be safely predicted that man will not develop the infection as long as his body fluids and tissues contain adequate quantities of the antitoxin.

The possibilities of this passive serum protection has natural limits of time, depending upon the rapidity of elimination of the foreign serum. The experimental data presented in this paper, which agree with the experience with antidiphtheritic and antitetanic antitoxins, indicate that, in all probability, a passive immunity to *Bacillus welchii* infection of at least 2 weeks' duration can be conferred upon man by a single injection of the antitoxin. This immunity will be sufficient in the majority of instances, since only sporadic cases of *Bacillus welchii* infection arise later than the 10th day after injury, and the greater number occurs within 48 hours of that time.

In the light of the results obtained in treating the infection in guinea pigs, it is reasonable to hope that the antitoxin will be of value also as a therapeutic agent. The indications are that early infectious cases can be readily arrested and the more advanced and severe ones ameliorated, if not wholly checked, so that surgical interference may be resorted to with greater probability of effectiveness.

The antitoxin in man should be administered intravenously and probably locally, about the wound, as well.

#### SUMMARY.

1. It has been possible to confer on guinea pigs a passive immunity of about 2 weeks' duration to *Bacillus welchii* toxin through a protective administration of the antitoxin.

2. Guinea pigs which had received a prophylactic dose of *Bacillus welchii* antitoxin exhibited pronounced resistance to infection with the virulent bacilli for a period of 12 days.

3. Established infections in guinea pigs with *Bacillus welchii* have been arrested and controlled by treatment with the antitoxin.

4. The opinion has been expressed that it will be possible to prevent *Bacillus welchii* infection in man through the prophylactic use of the antitoxin and developed cases of the infection may be controlled by therapeutic injections of the same agent.



# THE RELATION OF THE SPINAL CORD TO THE SPONTANEOUS LIBERATION OF EPINEPHRIN FROM THE ADRENALS.\*

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PLATES 44 TO 47.

(Received for publication, May 10, 1917.)

The demonstration of the complete dependence of the spontaneous liberation of epinephrin from the adrenals upon the integrity of their nerve supply naturally raises the question where the central mechanism which sustains this secretion is situated. We are not aware of the existence of evidence upon this point. Elliott's statement<sup>1</sup> that exhaustion of the store of epinephrin in the adrenals by electrical excitation of afferent nerves does not occur if the cord is transected anywhere below the level of the vasomotor center in the medulla oblongata, but does occur when transection of the brain-stem is made just above the anterior corpora quadrigemina, has no direct bearing on the question. For it has not been proved that the exhaustion of the store is due entirely or mainly to increase in the rate of liberation of epinephrin, on which Elliott made no observations. A change in the amount of the store of epinephrin would merely show that some alteration had occurred in the relation between the rate of formation and the rate of discharge of the epinephrin. Nor would such observations even if they were accepted as proving an increase in the liberation brought about reflexly through a center in the bulb or higher up, give any indication whether the steady spontaneous liberation of epinephrin is sustained from a center at this level. Absence of effect

\* A note on this work was published in the *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 143.

<sup>1</sup> Elliott, T. R., *J. Physiol.*, 1912, xliv, 407.

of afferent stimulation upon the epinephrin store after section of the cord just below the bulb likewise affords no indication whatever that the rate of spontaneous liberation of epinephrin has been interfered with. As a matter of fact, we have found in cats that after transection of the cord at various levels in the cervical region, the secretion of epinephrin into the blood, far from being abolished, proceeds without interruption. The rate of liberation may even remain sensibly the same, within the limits of error of the methods used for estimating it, as before the section. This is illustrated in Experiment 1, in which adrenal blood was obtained from a cava pocket before and after section of the cord opposite the body of the fourth cervical vertebra, between the fourth and fifth cervical segments.

*Experiment 1. Condensed Protocol.*—Cat; weight 2.31 kilos.

The animal received 4 gm. of urethane by stomach tube at 12.30 p.m., and 2 gm. at 2.00 p.m.

2.15 p.m. Tracheal and jugular cannulas were inserted and a specimen of jugular blood was obtained. The cord was exposed for about a segment in the midcervical region. A short cava pocket was then made, all the arteries (renal, celiac, and superior mesenteric, and abdominal aorta) being tied. Artificial respiration was started while the animal was breathing well spontaneously, and collection of blood from the adrenals begun.

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.
	<i>gm.</i>		<i>gm.</i>
1	2	20 sec.	6.6
2	10	2 min.	5.0
3	2.8	2 " 30 "	1.2
4	2.9	4 " "	0.7
5	2.4	4 " 15 "	0.6
6	4.5	6 " 10 "	0.7
7	3.3	7 " 10 "	0.5
8	2.1	7 " 10 "	0.3

After collection of the second adrenal specimen the cord was cut completely at the level of the body of the fourth cervical vertebra, between the fourth and fifth segments, as verified post mortem. After collection of the sixth adrenal specimen it was verified that the section of the cord was complete. After collection of the eighth specimen, while the pocket was still clipped off, blood was obtained from the abdominal aorta. The proportion of corpuscles in the blood (hematocrit) was 25.5 per cent. Combined weight of adrenals 0.440 gm.

In Figs. 1 to 3 are reproduced some of the tracings from the rabbit intestine and uterus segments on which the blood specimens were tested. At 2 (Fig. 1) the Ringer's solution in which the intestine segment was beating was replaced by indifferent (jugular) blood, and this at 3 by the second adrenal blood specimen, collected just before transection of the cord. The inhibition of the intestine, and therefore the concentration of epinephrin in the blood, was obviously much less than with the sixth adrenal specimen, collected after section of the cord (Observation 7). The inhibition produced by the sixth specimen was less than that produced by the eighth (Observation 10). The progressive increase in the concentration is associated with the progressive slackening in the blood flow in successive samples. This is a phenomenon always observed in animals with intact central nervous system when the rate of flow happens to be diminishing, and it is undoubtedly due to the fact that the rate of liberation of epinephrin per unit of time remains approximately constant, at least for considerable periods, under the experimental conditions. The mere inspection of these tracings is of itself sufficient to show that section of the cord at the level mentioned cannot have caused any very great change in the rate of liberation of epinephrin. Figs. 2 and 3 are some of the tracings taken to assay the concentration of epinephrin in the second adrenal blood specimen and in the eighth specimen, collected respectively before and after the cord section. The adrenalin used for the epinephrin assays was always freshly assayed colorimetrically. It was found that the concentration in the second specimen was approximately 1:13,000,000 (Fig. 2, Observations 36 and 38), an unusual degree of dilution corresponding to the unusually high rate of blood flow, associated with the high blood pressure before division of the cord, which is generally seen when all the arteries mentioned in the protocol have been ligated. The concentration in the eighth specimen was much greater than 1:1,600,000, and slightly less than 1:800,000 (Fig. 3, Observations 20, 22, and 24). The blood flow after section of the cord dropped abruptly, owing to paralysis of vasoconstrictors in spite of the previous ligation of arteries. It will be noted that the blood flow during collection of the eighth specimen was only one-sixteenth or one-seventeenth as great as during collection of the second specimen, while the concentration of epinephrin in the eighth specimen

was approximately sixteen times as great as in the second. In other words, the output of epinephrin (0.0004 mg. per minute) was not altered by the cord section.

It might be objected that the small blood flow during collection of the eighth specimen was not sufficient to prevent partial asphyxia of the adrenals, and that by some direct effect of this condition on the cells of the medulla an abnormal liberation of epinephrin, not mediated through the nervous system, took place. This objection is entirely without weight. For with even smaller flows through adrenals whose innervation has been interrupted no such liberation occurs.<sup>2</sup> After all, a flow of about 100 gm. of blood per 100 gm. of tissue, although small for the adrenal, is scarcely a starvation allowance.

It might much more plausibly be argued that the calculated output of epinephrin for the eighth specimen is likely to be less than the true rate after section of the cord, provided that the blood flow had not diminished. For it is quite unusual to find under any circumstances in adrenal blood collected in a cava pocket a much greater concentration than 1:1,000,000. If, then, with a declining rate of blood flow the maximum possible concentration has once been reached, the rate of liberation calculated for smaller flows will be less than the gland is capable of sustaining under the given experimental conditions with a more copious flow of blood. However, it is only when the calculated rate of liberation for the smaller flow is much less than for the larger flow, that any such question could arise. When with a relatively small blood flow the calculated output of epinephrin per minute is as great as with a larger flow all our experience goes to show that the rate of output calculated from the concentration and the blood flow can be legitimately compared for a wide range of blood flows lying above this smallest flow.

In the next experiment, the cord was transected at a slightly higher level between the third and fourth cervical vertebræ, through the fourth segment just above the origins of the fourth pair of cervical nerves, as shown at autopsy. The spontaneous liberation of epinephrin was studied by means of the eye reactions, the superior

<sup>2</sup> Stewart, G. N., and Rogoff, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 145; Marine, D., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1916-17, ix, 1.



cervical ganglion having been previously excised. The eye reactions were employed because they afforded a convenient means of investigating the effects of section of the nerves going to the adrenals upon the spontaneous liberation after section of the cervical cord.

*Experiment 2. Condensed Protocol.*—Cat; weight 2.19 kilos. Left superior cervical ganglion excised 6 days before the experiment.

9.30 a.m. 4 gm. of urethane given by stomach tube.

10.30 a.m. Tracheal cannula inserted and cervical cord exposed between the third and fourth vertebrae. Cava pocket made, abdominal aorta and renal vessels being tied.

11.15 a.m. Started artificial respiration.

Time.		Duration of pocket.	Pupil dilatation.
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#### ERRATUM.

On page 616, Vol. XXVI, No. 5, November 1, 1917, foot-note 2, for *Marine, D., and Rogoff, J. M., J. Pharm. and Exp. Therap., 1916-17, ix, 1*, read *Stewart, G. N., and Rogoff, J. M., J. Pharm. and Exp. Therap., 1917, x, 1*.

On page 627, foot-note 4, for *Stewart and Rogoff, J. Pharm. and Exp. Therap., 1916-17, ix, 479*, read *Stewart and Rogoff, J. Pharm. and Exp. Therap., 1916, viii, 479*.

On page 638, line 14, for *was interrupted* read *was never interrupted*.

On page 644, line 3, the reference number should be 5 instead of 4.

12.50	“	“	10	“	30	“	“
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The pocket filled very slowly throughout the experiment; the heart was feeble.

In this experiment, as will be seen from the protocol, section of the cord at the level mentioned caused no noteworthy change in the eye reaction, certainly no clear diminution. The circulation was poor both before and after the section. The cava pocket filled slowly and the interval between the release of the pocket and the beginning of

the pupil dilatation was correspondingly long. Yet approximately the same time of collection was required to evoke a reaction of given magnitude before and after section of the cord. After division of the splanchnics in the abdomen and the sympathetic trunks in the thorax no eye reactions could be obtained, even with much longer periods of collection in the pocket; that is, the liberation of epinephrin after section of the cord must have been sustained from some part of the cord below the fourth cervical segment, and through the same nerves as with intact central nervous system.

It may be explained here that even when the cava pocket has been kept closed for so long a period that it is unable to receive any more adrenal vein blood, there is good evidence that the output of epinephrin still goes on into the blood of the adrenal capillaries at an approximately constant rate; and that when the pocket is released the full effect of this epinephrin is exerted in eliciting the eye reactions or increasing the blood pressure, just as if the blood had actually passed into the pocket. So that in the course of an experiment, the reactions evoked when a pocket clipped off for a given length of time is released are substantially of the same magnitude, whether the pocket has been overfilled or underfilled in that time. Of course, the interval after which the reactions occur is greatly influenced by the rate of the circulation, since the epinephrin-containing blood released from the pocket will take longer to reach the reacting structures with a slow than with a rapid blood flow. With a very slow flow, also, the blood collected in a pocket may be so small in amount that it is not promptly or completely passed into the circulation on release of the pocket. It is further to be expected that below a certain rate of flow the function of the adrenal medullary cells, as already suggested, will be interfered with. In that case, the steadiness, for given conditions, in the output of epinephrin per unit of time, which is so strikingly manifested over a wide range in the rate of the blood flow, will no longer be maintained. Our experience shows, however, that this point is not easily reached.

In the next experiment transection was made about two segments lower in the cervical cord, in order to localize more sharply the level of the cord concerned in the spontaneous secretion of epinephrin.

*Experiment 3. Condensed Protocol.*—Cat; weight 3.175 kilos. Recent parturition. Left superior cervical ganglion excised 1 week before the experiment.

9.45 a.m. 4 gm. of urethane.

10.45 a.m. Tracheal cannula inserted and cervical cord exposed between fifth and sixth vertebræ. Cava pocket prepared; intestinal and renal arteries and abdominal aorta tied.

11.30 a.m. Left pupil wider than right, both nictitating membranes forward; cat breathing quite well, but for uniformity of observations artificial respiration was started.

Time.		Duration of pocket.	Pupil dilatation.	Reaction of nictitating membrane.
<i>a.m.</i>				
11.40	Pocket experiment.	1 min., 30 sec.	Doubtful.	None.
11.42	" "	2 " 30 "	None.	"
11.45	" "	3 " 45 "	"	"
11.50	" "	5 "	"	"
<i>p.m.</i>				
12.05	Pocket experiment.* Cord stimulated with needle electrodes one segment below the exposed part for 3 min.	3 "	"	"
12.15	Left sympathetic and vagus cut in neck.			
12.17	Right sympathetic cut in neck.			
12.20	Pocket experiment.	5 min., 30 sec.	Very good; in 10 sec.	Very good; 10 sec.
12.26	" "	1 "	Good; in 15 sec.	Good; 15 sec.
12.30	" "	2 "	Very good; in 10.8 sec.	Very good; 10.8 sec.
12.37	Spinal cord cut just below body of fifth cervical vertebra.			
12.41	Pocket experiment.	2 min.	Very good; in 15 sec.	Very good; 15 sec.
12.45	" "	1 "	Good; in 18.6 sec.	Good.

\* With each period of stimulation both pupils dilated instantaneously, and proportionally to the same extent, but the left pupil still remained wider than the right. After section of both cervical sympathetics, stimulation of the cord still caused dilatation of both pupils. At autopsy it was found that the cord had been cut between the fifth and sixth cervical segments.

Time.		Duration of pocket.	Pupil dilatation.	Reaction of nictitating membrane.
<i>p.m.</i>				
12.50	Major and minor splanchnics in abdomen cut on both sides.			
12.53	Pocket experiment.	2 min.	None.	None.
12.56	" "	5 "	Slight; in 20-25 sec.	"
1.05	Right semilunar ganglion excised; nerves coming to left ganglion cut. Circulation getting feeble.			
1.12	Pocket experiment.	5 min., 30 sec.	None.	None.
1.25	" "	9 " 30 "	Slight; in 60-90 sec.	Slight.
1.47	" "	24 "	Slight; in 40-45 sec.	
2.25	" "	25 "	None.	None.
3.05	" " with massage of adrenals.	15 "	"	"
3.30	Injected 0.5 cc. of 1:1,-600,000 adrenalin.		"	"
3.35	Injected 0.5 cc. of 1:270,000 adrenalin.		Slight; in 40-50 sec.	Slight.

In this animal the denervated eye reactions were employed and an interesting preliminary observation was made upon them without some reference to which the first part of the protocol would probably appear as puzzling to the reader as the observations did to us when they were being made. The left superior cervical ganglion had been excised a week before the experiment. The eye reactions ought, therefore, to have been easily obtained in the pocket experiments made between 11.40 and 12.05 before section of the cord. In the numerous observations made by us, we have, apart from this experiment, scarcely ever had a negative result, especially with a duration of occlusion of the pocket as long as 5 minutes, and with the good blood flow and satisfactory filling of the pocket which existed in this cat. It was conceivable, of course, that for some reason the adrenals might have been giving off much less epinephrin than usual; or that the reactions of the iris, etc., might have been unduly depressed by

the anesthetic, although there was nothing in the behavior of the animal to indicate that the urethane, a very uniform anesthetic, as is well known, had affected this animal at all differently from any of the others. Desiring to increase the output of epinephrin to the maximum, we stimulated the cord with needle electrodes, hoping thus to strike the secretory path, but again with a negative result as regards the eye reaction. Even with the collection of adrenal blood in the pocket for as long as 3 minutes during stimulation no eye reactions were obtained. Stimulation of the cord, however, caused immediate dilatation of the pupils of both eyes. This dilatation was still elicited after section of both vago-sympathetics in the neck, and may be attributed to stimulation of afferent fibers in the cord. But the interesting point was that after section of the cervical sympathetics excellent eye reactions were now evoked with collections of adrenal blood in the cava pocket much shorter than those which gave a negative result before division of the nerves. The most probable conclusion would seem to be that some small part of the superior cervical ganglion had escaped excision, and that the innervation of the eye through the cervical sympathetic was not entirely interrupted, although it was impossible to verify this at autopsy on account of scar tissue. The change in the sensitive structures of the iris and nictitating membrane, on which the increased power of reaction to adrenalin depends, must be assumed to have developed as usual after removal of the ganglion, although prevented from manifesting itself, even in the presence of a quantity of epinephrin more than sufficient to evoke good reactions, until the control of the remaining sympathetic fibers was removed.

It will be seen from the protocol that after section of the cord between the fifth and sixth cervical segments excellent pupil and nictitating membrane reactions were still obtained. The reactions were not noticeably less for equal periods of collection of blood in the pocket than before the section, although the interval after which they occurred was somewhat lengthened, corresponding to the slower blood flow. Subsequent division of the major and minor splanchnics in the abdomen greatly weakened the reactions and increased the time of collection necessary to elicit even a feeble response. Further section of fibers coming to the adrenals abolished the reactions even

with very long periods of collection. This was not due to total loss of sensitiveness of the reactive structures in the eye. For even at the end of the experiment a slight but definite response was still obtained on injection of 0.002 mg. of adrenalin.

As a first approximation towards defining the lower limit of the region of the cord concerned in the spontaneous liberation of epinephrin the following experiment was performed, both eye reactions and rabbit segment tests being employed.

*Experiment 4. Condensed Protocol.*—Cat; weight 1.72 kilos. Left superior cervical ganglion excised 8 days before the experiment.

10.00 a.m. 3.5 gm. of urethane.

11.20 a.m. Tracheal cannula inserted. Cervical and dorsal cord exposed for about a segment at each point. Long cava pocket made and arteries (renal, celiac, mesenteric, and abdominal aorta) tied.

Time.		Duration of pocket.	Pupil dilatation.	Reaction of nictitating membrane.
<i>p.m.</i>				
12.15	Pocket experiment.	1 min., 10 sec.	Good; in 8.8 sec.	Positive; 11 sec.
12.18	“ “	2 “	Very good; in 8.8 sec.	Very good; 8.8 sec.
12.22	Cut cord between fifth and sixth cervical vertebrae.			
12.32	Pocket experiment.	1 min., 45 sec.	Very good; in 10.8 sec.	Very good (a little later).
12.35	“ “	2 “	Very good; in 11.2 sec.	Very good (a little later).
12.40	Cut cord at fourth dorsal vertebra.			
12.49	Pocket experiment.	2 min.	None.	None.
12.55	“ “	3 “	“	“
	In the last two pocket observations the filling was slower than before.			
1.07	Pocket experiment with stimulation of cord between fifth and sixth dorsal vertebrae.	3 min.	Very good; in 20.2 sec.	Very good; 20.2 sec.

1.20 p.m. Put cannula in lower end of cava, making a short pocket, and collected two specimens of adrenal blood. First specimen, 1.6 gm. in 5 min.,

45 sec. (0.3 gm. per min.); second specimen, 3.7 gm. in 20 min. (0.2 gm. per min.). With the pocket still clipped off, blood was obtained from the abdominal aorta.

The left adrenal weighed 0.214 gm. and contained 0.12 mg. of epinephrin; the right adrenal weighed 0.238 gm. and contained 0.12 mg. of epinephrin.

The experiment shows that after transection of the cord between the fifth and sixth cervical vertebræ (through the fifth cervical segment, just below the fifth pair of nerve roots, as found at autopsy) the eye reactions were elicited by the adrenal blood, apparently in the same strength for a given time of closure of the cava pocket as before the section, although the interval between release of the pocket and the beginning of the pupil dilatation was somewhat lengthened, as would necessarily be the case owing to the lowered blood pressure and diminished speed of the blood. When the cord was now cut between the fourth and fifth dorsal vertebræ (between the third and fourth segments, as shown at autopsy) no eye reactions could be obtained even with longer periods of occlusion of the pocket than sufficed to cause excellent reactions just before. That the negative result was not due to diminished blood flow, but that the adrenals were still capable of secreting epinephrin actively, was proved by stimulating the cord electrically by needle electrodes inserted one above the fifth, and the other above the sixth dorsal spine while the adrenal blood was being collected in the pocket. Very good eye reactions followed the opening of the pocket, naturally after a longer interval than before the dorsal section, corresponding to the slower blood flow. The adrenal blood specimens now drawn off were so small that it was not quite certain whether some of the epinephrin in them might not have been liberated during the manipulations in inserting the cannula. Despite this, however, the concentration found even in the first specimen was somewhat less than 1:17,000,000, corresponding to an output of epinephrin per minute of 0.00002 mg.; that is, far below any concentration or output ever met with in the cat with intact adrenal innervation.

The experiments next to be considered, in which the animals were allowed to survive 2 or 3 days after the cord section<sup>3</sup> before the epinephrin output was tested, so that any possible irritative discharge

<sup>3</sup> All the operations were performed under ether anesthesia.

might be eliminated, yield clear evidence that transection of the dorsal cord at the level mentioned in the last experiment, or even a segment higher reduces the rate of liberation almost to zero. Transection one segment lower abolished the liberation entirely, or at least reduced it so much that no epinephrin could be detected in the adrenal blood by sensitive rabbit intestine and uterus segments. The upper limit of the portion of the cord related to the spontaneous liberation of epinephrin was also further defined by survival experiments and these may be taken first.

*Experiment 5. Condensed Protocol.*—Cat; weight 2.17 kilos. Cord transected at the level of the body of the last cervical vertebra 3 days before the experiment. Animal in fairly good condition. Anesthetized with ether for the insertion of the tracheal cannula; thereafter no more ether was required as the operative field was, of course, absolutely anesthetic because of the previous spinal cord section. Cava pocket made with ligation of all the usual arteries. Cannula inserted in lower end of pocket (short pocket) and the following samples of adrenal blood collected.

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.
	<i>gm.</i>		<i>gm.</i>
1	2.4	4 min., 50 sec.	0.5
2	2.2	7 " 30 "	0.3
3	2.1	9 "	0.23
4	2.2	17 " 30 "	0.13

While the pocket was still clipped off blood was obtained from the abdominal aorta. While the pocket was being tied off some blood was left in it and the first adrenal specimen was therefore somewhat diluted. The autopsy showed that the cord had been divided between the last cervical and the first thoracic segments. Combined weight of adrenals 0.362 gm.

In survival experiments it is not so easy as in acute experiments to decide whether transections of the cervical cord leave the rate of liberation of epinephrin unaltered, or somewhat diminish it because the rate before and after the section cannot be compared on the same animal within a short interval of time. All that can be done is to determine whether the residual output after the cervical section is within the range established for animals under the same experimental conditions, but with intact central nervous system. In Experiment



5, as will be seen from the specimens of rabbit intestine and uterus tracings reproduced in Figs. 4 to 6, concentrations of epinephrin within the normal range<sup>2</sup> were found in the adrenal blood samples collected after section of the cervical cord between the last cervical and the first thoracic segments.

The adrenalin assays on the intestine segments gave a concentration, in the fourth adrenal specimen, of about 1: 1,500,000 (Fig. 4, Observation 19; Fig. 5, Observation 27); and in the second adrenal specimen a concentration greater than 1: 2,500,000 and less than 1: 1,500,000 (Fig. 4, Observation 21; Fig. 5, Observations 29 and 27). Taking the concentration in the second specimen as 1: 2,000,000, we get a liberation of epinephrin per minute of not quite 0.0002 mg. (0.0001 mg. per kilo of body weight). It may be considered certain that the output which the glands would have been capable of maintaining with a more nearly normal blood flow was at any rate not less than this. It might have been more, since as already mentioned, we rarely encounter concentrations of more than 1: 1,000,000 in adrenal blood collected under our experimental conditions; and therefore the point might already have been passed at which the declining blood flow can be compensated by increased concentration of epinephrin.

The fact that it was not necessary to administer an anesthetic in order to collect the adrenal blood in this animal, since the cord section had rendered the operative field totally insensitive, has probably no bearing on the question whether the rate of output was somewhat diminished by the cervical section. For although some writers have assumed that anesthetics markedly increase the rate of liberation of epinephrin, there is no real proof of this. In any case, if the anesthetic exerts its effect through the higher parts of the central nervous system, the administration of an anesthetic could not have increased the output of epinephrin in this animal. In reference to the experiments (Nos. 1 to 4) in which the epinephrin output was determined immediately after section of the cervical cord, it might be asked, however, whether the anesthetic had not already abolished the activity of any portion of the brain or bulb which might be related to the epinephrin secretion. Division of the cervical cord would in that case cause no diminution in the output of epinephrin if made above the level of the spinal center. An assumption equally plausible, but at present equally devoid of experimental basis, is that anesthetics abolish or lessen an inhibition of the spinal center from a center in the brain. On this hypothesis, the output of epinephrin seen after cervical cord section would be considered as greater, not smaller, than the normal output with intact central nervous system.

The progressive increase in the concentration of epinephrin in successive adrenal blood samples associated with gradual diminution in the rate of blood flow is well brought out in the intestine tracings reproduced in Fig. 4, and still better in the uterus tracings in Fig. 6. This phenomenon and what underlies it—the stability in the rate of epinephrin discharge—is so characteristic when adrenal blood is collected with intact central nervous system, that its occurrence after spinal section lends support to the conclusion that the secretion of epinephrin when the connection of the cord with the brain has not been interrupted is also sustained largely, if not entirely, from the cord.

In the next experiment the eye reactions were studied in a cat 2 days after section of the cord just above the body of the seventh cervical vertebra.

*Experiment 6. Condensed Protocol.*—Cat; weight 2.825 kilos. Left superior cervical ganglion excised 1 week, and spinal cord divided 2 days before the experiment, just above the body of the last cervical vertebra.

10.00 a.m. Anesthetized with ether while the tracheal cannula was being inserted; thereafter no more ether was required as the operative field was necessarily absolutely insensitive because of the previous section of the cord.

10.05 a.m. The abdomen was opened and the cava pocket made, all the usual arteries being tied.

Time.		Duration of pocket.	Pupil dilatation.	Reaction of nictitating membrane.
a.m.				
10.28	Pocket experiment.	1 min.	Small; in 20 sec.	Small (shortly after pupil).
10.30	“ “	2 “	Good; in 15 sec.	Good; 15 sec.
10.45	Injected 0.5 cc. (1:330,000) of adrenalin into jugular vein.		Very good; in 15 sec.	Very good; 15 sec.
10.50	Injected 0.25 cc. (1:330,000) of adrenalin into jugular vein. The eye reactions after this injection were about the same or slightly less than in the observation at 10.30 a.m.		Good; in 17.2 sec.	Good; 17.2 sec.
10.55	Pocket experiment.	1 min.	Very slight; in 25 sec.	None.
10.58	“ “ The reactions were about the same as at 10.50 a.m.	2 “	Good; in 21 sec.	Good; 21 sec.

Time.		Duration of pocket.	Pupil dilatation.	Reaction of nictitating membrane.
<i>a.m.</i>				
11.02	Pocket experiment. The reactions were about the same as at 10.45 a.m.	3 min., 40 sec.	Very good; in 16.8 sec.	Very good; 16.8 sec.
11.10	Cut nerves to both semilunar ganglia in abdomen.			
11.20	Pocket experiment. Pocket not so well filled as before.	3 min.	None.	None.
11.25	Pocket experiment. The reaction was about the same as at 10.58 a.m.	5 "	Distinct; in 23.4 sec.	Distinct.
11.30	Both semilunar ganglia extirpated.			
11.37	Pocket experiment.	5 min.	None.	None.
11.45	" " Pocket filled very slowly.	8 "	Slight; in 41 sec.	"
11.55	Cut lumbar sympathetic chain just below diaphragm.			
12.00	Pocket experiment. Very poor flow.	25 min.	Slight; in 90 sec.	None.
<i>p.m.</i>				
12.50	Pocket experiment. Better flow.	10 "	Slight; in 60 sec.	"

The autopsy showed that the cord was divided through the seventh cervical segment, immediately below the origins of the seventh pair of nerves.

The fact that adrenal blood collected in the cava pocket caused good eye reactions was ascertained. It was shown by the injection of adrenalin solution that the amount of epinephrin secreted per minute was about 0.0004 mg. (0.00015 mg. per kilo of body weight per minute); this is a substantial output, although considerably less than the average, as estimated by eye reactions, in cats with intact central nervous system.<sup>4</sup> Various nerves going to the adrenals were then divided and the effect in diminishing the eye reactions was noted. After division of the fibers coming to the semilunar ganglia, the eye reactions elicited by adrenal vein blood collected in the cava pocket were markedly diminished. A 3 minute collection gave no reactions, whereas before the nerve section a 1 minute collection caused a slight

<sup>4</sup> Stewart and Rogoff, *J. Pharm. and Exp. Therap.*, 1916-17, ix, 479.

effect on the pupil, and a 2 minute collection good dilatation of the pupil and retraction of the nictitating membrane. After removal of both semilunar ganglia, which, of course, insured the section of any strands coming to the ganglia overlooked in the previous section, a 5 minute collection of adrenal blood caused no eye reactions, although previously a 2 minute collection gave good reactions. On release of a pocket occluded for 8 minutes, a small dilatation of the pupil was obtained.

In connection with the fact that slight eye reactions were still elicited with long periods of closure of the cava pocket even after extensive section of possible nerve paths to the adrenal glands, it must be pointed out that these nerve sections entail considerable manipulation of, and in the neighborhood of, the adrenals. With the slow blood flow toward the end of the experiment, epinephrin liberated by massage would take long to be completely washed out. It may be concluded that even the feeble reactions obtained after these nerve sections were not due entirely to genuinely secreted epinephrin. Survival experiments published elsewhere<sup>2</sup> have shown that the epinephrin output after section of the adrenal nerves is either abolished or reduced so much as to be incapable of detection by sensitive rabbit intestine and uterus segments. On the other hand, in acute experiments after the same nerve sections, although the output of epinephrin is greatly reduced, a content capable of detection is usually still found in adrenal blood.

It may, therefore, be confidently assumed that the whole output of epinephrin from the adrenals after section of the cervical cord near its lower limit is mediated through the same nerves which are concerned in the liberation with intact nervous system.

The last two survival experiments to be quoted were made for the purpose of defining more exactly the lower limit of the spinal region concerned in the epinephrin secretion.

*Experiment 7. Condensed Protocol.*—Cat; weight 1.59 kilos. Left superior cervical ganglion excised 6 days before the experiment and spinal cord transected between the fifth and sixth thoracic vertebræ 3 days before the experiment. Condition good. The autopsy showed cord section between the fifth and sixth thoracic segments.

2.00 p.m. Ether was given while the tracheal and jugular cannulas were

inserted. Thereafter no more ether was required as the operative field was, of course, totally insensitive owing to the cord section. A specimen of jugular blood was obtained. A long cava pocket was prepared; the renal arteries and abdominal aorta were tied.

2.45 p.m. Pocket experiment, 1 minute, 25 seconds. No eye reactions.

2.48 p.m. Pocket experiment, 3 minutes, 20 seconds. No eye reactions.

The blood flow was good; the pocket filled well.

3.00 p.m. Intestinal arteries tied. Cannula put in lower end of pocket (now made into a short pocket). Adrenal blood specimens collected as follows:

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.
	<i>gm.</i>		<i>gm.</i>
1	1.5	1 min., 25 sec.	1.0
2	3.2	4 " 30 "	0.7
3	3.5	10 "	0.35
4	2.2	10 "	0.22

Through a cannula in the jugular vein inserted under ether anesthesia the following injections were made: 0.5 cc. (1:500,000) of adrenalin; good pupil and nictitating reactions in 15 seconds. 0.2 cc. (1:500,000) of adrenalin; small nictitating reaction in 17 seconds; small pupil reaction in 25 seconds. While the pocket was still clipped off blood was obtained from the abdominal aorta.

Left adrenal weighed 0.206 gm. and contained 0.20 mg. of epinephrin; the right adrenal weighed 0.194 gm. and contained 0.18 mg. of epinephrin.

In this cat 3 days after transection of the cord between the fifth and sixth thoracic segments no eye reactions could be evoked by adrenal blood even with relatively long periods of collection in the cava pocket, periods which would certainly have given good reactions either with intact central nervous system or with the cord cut in the cervical region. The reactions were not lacking because the iris and nictitating membrane were incapable of responding to small quantities of epinephrin. For even at an advanced period in the experiment, after the withdrawal of several samples of adrenal blood through a cannula in the cava, good eye reactions were obtained on injection of 0.001 mg. of epinephrin, and quite detectable reactions on injection of 0.0004 mg. Tested with rabbit intestine and uterus segments, the adrenal blood gave a negative result (Fig. 7, Observation 27; Fig. 8, Observations 6 and 12), although a concentration of epinephrin of 1:60,000,000 (Fig. 8, Observation 37) could easily have

been detected by the intestine. There was evidence that the adrenal blood (third specimen) could not have contained even 1:100,000,000 epinephrin, corresponding to an output of at most 0.000003 mg. per minute, not one-hundredth of the output to be expected in a normal cat under the experimental conditions. It must be repeated that there was no evidence that any epinephrin was being discharged by the adrenals. Nothing could be more striking than the contrast between the concentration and output per minute of epinephrin in this cat and in those whose spinal cord was transected towards the lower level of the cervical region. For instance, the concentration in the second adrenal specimen in Experiment 5 was 1:2,000,000; and the calculated output per minute, 0.0002 mg. Even when the cord was divided (Experiment 8) two segments higher in the dorsal region, through the third thoracic segment, although a slight epinephrin liberation was detected by intestine segments, the output was enormously reduced (to 0.000006 mg. per minute).

*Experiment 8. Condensed Protocol.*—Cat; weight 3.65 kilos. Left superior cervical ganglion excised 10 days before the experiment. Spinal cord transected between the third and fourth dorsal vertebræ 3 days before the experiment. Condition excellent. Ether anesthesia was used throughout the experiment. Long cava pocket prepared in the usual manner, renal, celiac, and mesenteric arteries, and abdominal aorta being tied.

Time.		Duration of pocket.	Pupil dilatation.	Reaction of nictitating membrane.
<i>a.m.</i>				
11.00		1 min., 20 sec.	None.	None.
11.05		1 " 50 "	"	"
11.10		3 "	"	"
11.15		5 "	"	"
11.30	Injected 0.5 cc. (1:530,000) of adrenalin into jugular vein.		Excellent; in 10.6 sec.	Excellent; 10.6 sec.
11.35	Injected 0.2 cc. (1:530,000) of adrenalin into jugular vein.		Very good; in 14.8 sec.	Very good; 14.8 sec.
11.38	Injected 0.5 cc. (1:2,000,000) of adrenalin into jugular vein.		Small; in 22 sec.	None.
11.40	Injected 0.5 cc. (1:2,000,000) of adrenalin into jugular vein.		Small; in 22 sec.	"

11.55 a.m. Obtained indifferent blood from the other jugular.

12.00 m. Inserted cannula in cava (short pocket) and collected adrenal blood as follows:

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.
	<i>gm.</i>		<i>gm.</i>
1	3.6	2 min.	1.8
2	8.8	6 "	1.46
3	5.0	6 "	0.83
4	5.5	7 " 30 sec.	0.73
5	4.0	6 "	0.66

While the pocket was still clipped off a second specimen of indifferent blood (jugular) was obtained. The autopsy showed that the spinal section (between the third and fourth dorsal vertebræ) was through the origins of the third pair of thoracic nerves. The bloods were tested on rabbit segments. The right adrenal weighed 0.200 gm. and contained 0.18 mg. of epinephrin; the left adrenal weighed 0.208 gm. and contained 0.18 mg. of epinephrin.

The concentration of epinephrin in the third adrenal specimen (Fig. 9, Observation 30) was distinctly less than 1:135,000,000 (Observation 32), and very much less than 1:70,000,000 (Observation 28). The concentration in the fifth adrenal specimen was somewhat greater than 1:135,000,000. The eye reactions were negative, even when the cava pocket was closed for as much as 5 minutes, although the injection of 0.00025 mg. of epinephrin gave a definite pupil dilatation, and the injection of 0.0004 mg. a very good dilatation of the pupil and retraction of the nictitating membrane.

The results of the whole series of experiments are singularly consistent, particularly in view of the fact that there has been no selection of experiments. The eight experiments comprise all those performed, except one which has not been reported because the animal died before it was satisfactorily completed.<sup>5</sup> The results indicate

<sup>5</sup> In this animal the cord was divided between the second and third thoracic vertebræ, just below the origins of the second thoracic nerves, or nearly a segment higher than the highest dorsal section in the experiments reported. 3 days afterwards the eye reactions (the left superior cervical ganglion had been excised a week before the experiment) were found negative with collections in the cava pocket up to more than 3 minutes, although very good pupil and nictitating reactions were elicited by injection of 0.0015 mg. of epinephrin, and slight reactions by the injection of 0.0006 mg. The blood flow at the time of these observations was quite satisfactory, but the animal died be-

clearly that there exists in the cord between the last cervical segment and the fourth thoracic segment a mechanism which sustains the output of epinephrin from the adrenal glands after the cord is severed from the higher parts of the central nervous system. The experiments prove definitely that the center does not extend lower than the thoracic segment mentioned, and that at least an important part of it lies below the level of the last cervical segment. The possibility, however, is not excluded that the center may extend for some distance above the last cervical segment. It is of interest in connection with the currently accepted view of the development of the adrenal medulla, that the portion of the cord at which the sympathetic outflow begins should be identified as a center controlling the liberation of the only constituent of its secretion hitherto definitely recognized. If epinephrin in the quantities and concentrations in which it appears in the adrenal blood could be shown to fulfil an important office in maintaining the function of the sympathetic by activating certain of its elements, or by heightening or prolonging the effects resulting from its excitation, the location of an epinephrin center in the sympathetic region of the cord might perhaps acquire a new significance.<sup>6</sup> It might

fore adequate adrenal blood samples could be drawn off from the cannula in the cava. The small quantity of adrenal blood obtained while the blood was flowing very slowly showed a good concentration of epinephrin as tested on the rabbit segments. Since the eye reactions cannot in general detect outputs of epinephrin easily detectable by the rabbit segments, this result, although no great stress can be laid upon it in the absence of better samples of adrenal blood, is quite consistent with the general conclusion deduced from the other experiments as to the position and limits of the portion of the cord concerned in epinephrin secretion from the adrenals.

<sup>6</sup>It is difficult to demonstrate that the epinephrin spontaneously liberated from the adrenals has any effect upon the blood pressure unless its action is accumulated by collecting the adrenal blood in a cava pocket and then releasing it. The majority of recent observers have not seen any change in the blood pressure when the adrenal veins are carefully clipped. On the denervated eye, however, we have observed a phenomenon which indicates that even the small concentrations of epinephrin which can exist in the capillary blood when the adrenal blood is passing steadily into the circulation without being accumulated in the cava pocket can produce a demonstrable effect upon these extraordinarily sensitive objects. When the pupil has been dilated or the nictitating membrane retracted by release of adrenal blood collected in the cava pocket, the dilatation



then be permissible to speculate upon the possibility that the relative constancy of the epinephrin discharge, so puzzling on the hypothesis that it directly influences physiological events in virtue of the truly gross changes necessary to produce a detectable hyper- or hypoadrenalinemia, is associated with a more general and permanent action upon the sympathetic mechanisms, which does not entail the necessity of abrupt outbursts and remissions in the rate of liberation. To employ a simile which is doubtless excessively crude: if epinephrin is not the horse in the sympathetic machine, which must go now faster, now slower; nor even the whip which must sometimes be wielded vigorously and then be laid aside, is it not perhaps the lubricant which, whether the axle turns fast or slow, need not vary much in amount?

The possibility must, however, not be lost sight of, that epinephrin although the first definite constituent of the adrenal secretion to be discovered is not the only, nor the most important one which exists. It is difficult to conceive of a nervous control so complete as that which governs the output of epinephrin being developed in the case of a substance functionally unimportant. Yet, as we have shown in another place,<sup>2</sup> the output of epinephrin from the adrenals in cats is greatly and permanently reduced or abolished by section of the adrenal nerves without apparently interfering with the life or health of the animal. Section of the dorsal cord which, as has been shown above, produces a similar effect upon the output of epinephrin is also well known to be compatible with good health and long survival. Is there perhaps some as yet unknown substance of more importance than epinephrin which is normally given off from the adrenals under the influence of nerves, the secretion of which is eventually resumed after the nerves have been severed?

#### SUMMARY.

1. After section of the spinal cord in cats in the cervical region, as low as the last cervical segment, epinephrin continues to be liberated from the adrenal glands. This liberation has all the characters of the pupil disappears more slowly and the nictitating membrane comes forward more gradually when the pocket is left open than when it is clipped. Obviously, the steadily liberated epinephrin exerts an effect in prolonging the reactions once they have been elicited.

the normal secretion with intact central nervous system. It is sustained through the same nerve paths connecting the cord with the adrenals.

2. After section of the cord in the middorsal region the spontaneous liberation of epinephrin from the adrenals is abolished within the limits of detectability by the methods employed (denervated eye reactions of Meltzer, and rabbit intestine and uterus segments).<sup>7</sup>

3. The portion of the cord concerned in the liberation of epinephrin does not appear to extend much below the third thoracic segment.

4. In acute experiments on cats under urethane anesthesia no change in the rate of liberation of epinephrin, which could be detected by the tests employed, was observed when the cord was severed in the cervical region.

#### EXPLANATION OF PLATES.

##### PLATE 44.

FIG. 1. Intestine tracings. Blood from a cat. At 2 Ringer's solution was replaced by jugular blood, and this at 3 by the second adrenal blood specimen, collected with intact spinal cord. At 6 and 9 Ringer's solution was replaced by jugular blood, and this at 7 and 10 by the sixth and eighth adrenal blood specimens respectively, collected after section of the cord between the fourth and fifth cervical segments. All the bloods were diluted with four volumes of Ringer's solution. Reduced one-third.

FIG. 2. Intestine tracings. Blood of the same cat used for Fig. 1. At 35 Ringer's solution was replaced by arterial blood, and this at 36 by the second adrenal blood specimen, collected before section of the cord. At 43 Ringer's solution was replaced by arterial blood, and this at 44 by the third adrenal blood specimen, collected just after transection of the cord between the fourth and fifth cervical segments. All the bloods were diluted with three volumes of Ringer's solution. At 37 Ringer's solution was replaced by arterial blood diluted with three volumes of Ringer's solution, and this at 38 by arterial blood to which adrenalin had been added to make up a concentration of 1:13,000,000, the adrenalin blood being then diluted with three volumes of Ringer's solution. Reduced one-third.

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<sup>7</sup> We have since found that semisection of the cord (between the fourth and fifth dorsal segments in one cat, between the third and fourth segments in another) abolished the liberation of epinephrin from the adrenal of the same side without affecting the liberation from the other adrenal.

## PLATE 45.

FIG. 3. Intestine and uterus tracings. Blood of the same cat used for Figs. 1 and 2. At 19 Ringer's solution was replaced by jugular blood, and this at 20 by the eighth adrenal blood sample, collected after section of the cord between the fourth and fifth cervical segments. Both bloods were diluted with eight volumes of Ringer's solution. At 21 and 23 Ringer's solution was replaced by adrenalin in jugular blood, made up to 1:1,600,000 and 1:800,000, respectively, and then diluted with eight volumes of Ringer's solution. 4, 5, and 6 are uterus tracings. At 4 Ringer's solution was replaced by the eighth adrenal blood specimen, at 5 by the second, and at 6 by indifferent (arterial) blood; all were diluted with seven volumes of Ringer's solution. Reduced one-third.

FIG. 4. Intestine tracings. Adrenal blood from a cat after section of the cord between the last cervical and first thoracic segments. At 18, 20, and 22 Ringer's solution was replaced by indifferent (arterial) blood, and this at 19, 21, and 23 by the fourth, second, and third adrenal specimens, respectively. All the bloods were diluted with two volumes of Ringer's solution. Reduced one-half.

FIG. 5. Intestine tracings showing some of the adrenalin assays for the adrenal bloods used for Fig. 4. At 26 and 28 Ringer's solution was replaced by indifferent (arterial) blood, diluted with two volumes of Ringer's solution and this at 27 and 29 by adrenalin in arterial blood, made up to 1:1,500,000 and 1:2,500,000, respectively, and then diluted with two volumes of Ringer's solution. Reduced one-half.

## PLATE 46.

FIG. 6. Uterus tracings. Blood of the same cat used for Figs. 4 and 5. At 36 Ringer's solution was replaced by the third adrenal specimen; at 37, by the second, both diluted with eight volumes of Ringer's solution. The third specimen has a stronger effect than the second, corresponding to the slower blood flow during its collection. But as the increase of tone produced by the second specimen even was nearly maximal, greater dilution was necessary to show the difference clearly. At 38 Ringer's solution was replaced by the second adrenal specimen, and at 39 by the third, both diluted with twelve volumes of Ringer's solution. The difference is now evident. At 40 Ringer's solution was replaced by the fourth, and at 41 by the third adrenal specimen, both diluted with sixteen volumes of Ringer's solution. The increase of tone produced by the third specimen was now so nearly maximal for the condition of the segment at the time that greater dilution was resorted to, to bring out the difference, clearly seen in Observations 43 and 44, where Ringer's solution was replaced by the third and fourth specimens respectively, both diluted with twenty-four volumes of Ringer's solution. At 42 Ringer's solution was replaced by indifferent (arterial) blood, diluted with twenty-four volumes of Ringer's solution. Reduced one-half.

FIG. 7. Intestine tracings. Adrenal blood from a cat after section of the cord between the fifth and sixth dorsal segments. At 25 Ringer's solution was replaced by indifferent (arterial) blood, and this at 27 by the third adrenal specimen, both bloods being undiluted. The magnification is high, nearly twice as great as in Fig. 8, in order to afford the best chance for an inhibitory effect to be seen on the tracing. The writing point went somewhat above the drum after the addition of the adrenal blood and no inhibition was produced. To save space, the tracing has been cut horizontally. To reconstruct it, the right-hand portion may be imagined to be pushed up to the top of the figure and then to the left till it is in line with the left-hand portion.

PLATE 47.

FIG. 8. Intestine tracings. Blood from the same cat as in Fig. 7, but with a smaller magnification. At 5 and 11 Ringer's solution was replaced by arterial blood, and this at 6 and 12 by the fourth and the third adrenal specimens, respectively, the bloods being diluted with four volumes of Ringer's solution. At 34 and 36 Ringer's solution was replaced by arterial blood (undiluted), and this at 35 and 37 by adrenalin in arterial blood (1:40,000,000 and 1:60,000,000 respectively). The adrenalin blood was undiluted. Reduced one-third.

FIG. 9. Intestine tracings. Adrenal blood from a cat after section of the cord through the third thoracic segment. At 29 Ringer's solution was replaced by jugular blood, and this at 30 by the third adrenal blood specimen. At 27 and 31 Ringer's solution was replaced by jugular blood, and this at 28 and 32 by jugular blood to which adrenalin had been added to make up a concentration of 1:70,000,000 and 1:135,000,000, respectively. All the bloods were undiluted. Reduced one-third.

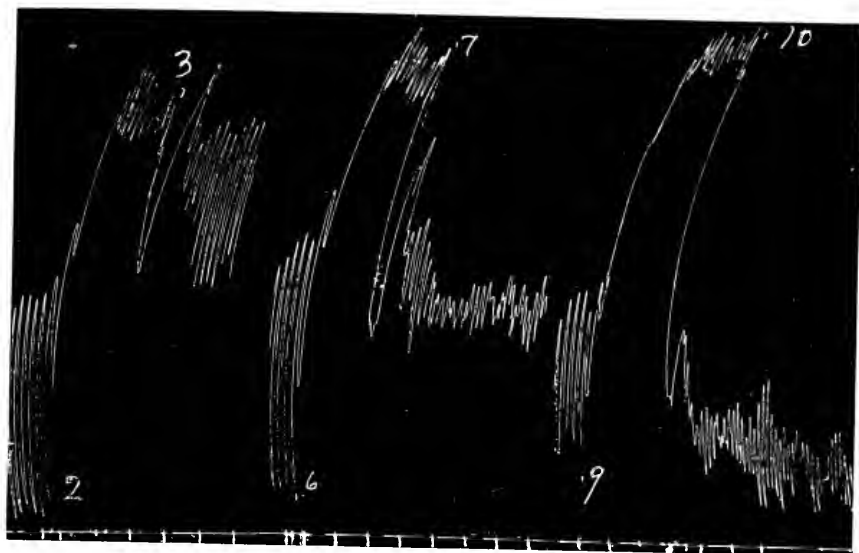


FIG. 1.

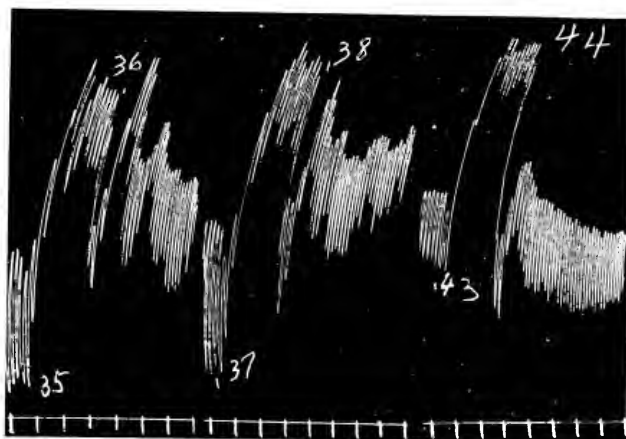
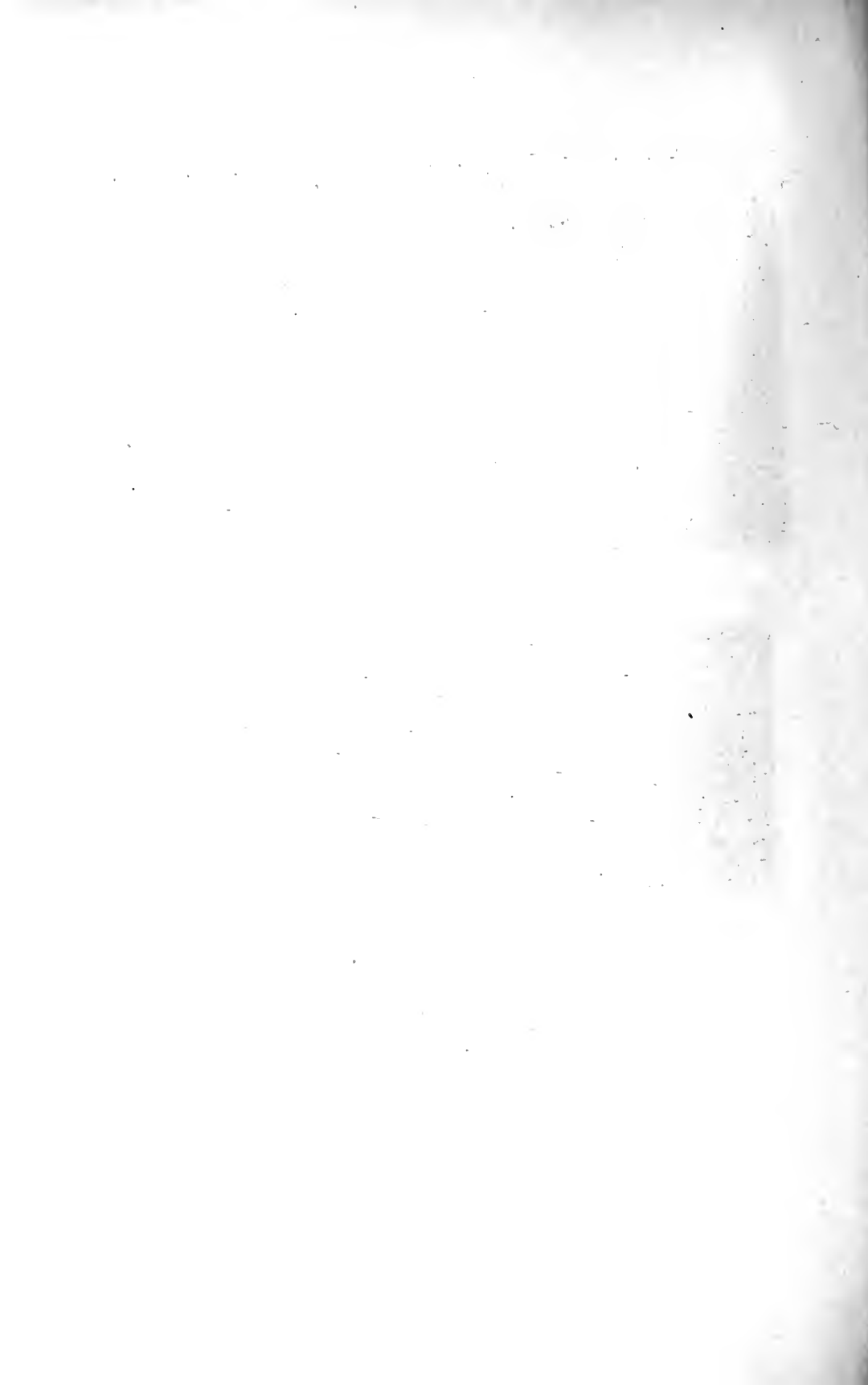


FIG. 2.

(Stewart and Rogoff: Spontaneous liberation of epinephrin.)



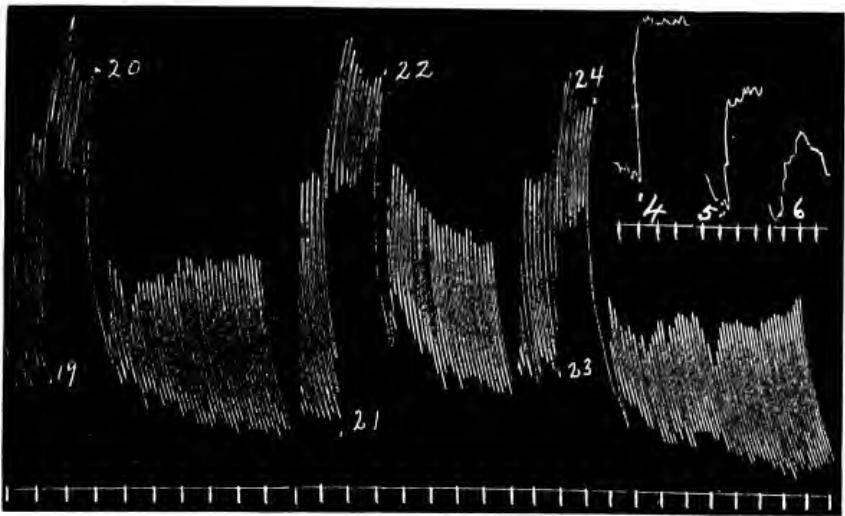


FIG. 3.

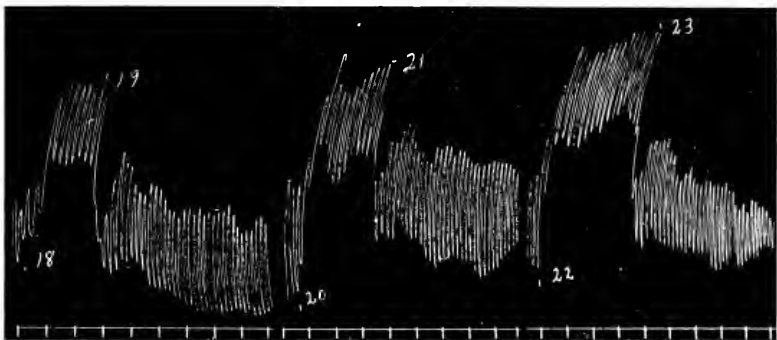


FIG. 4.

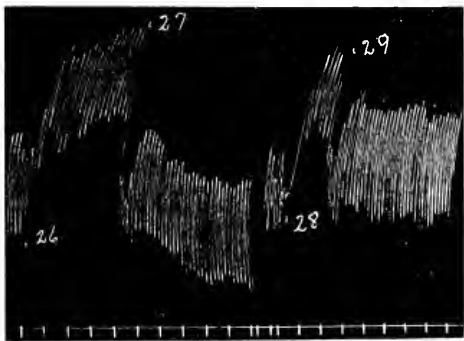
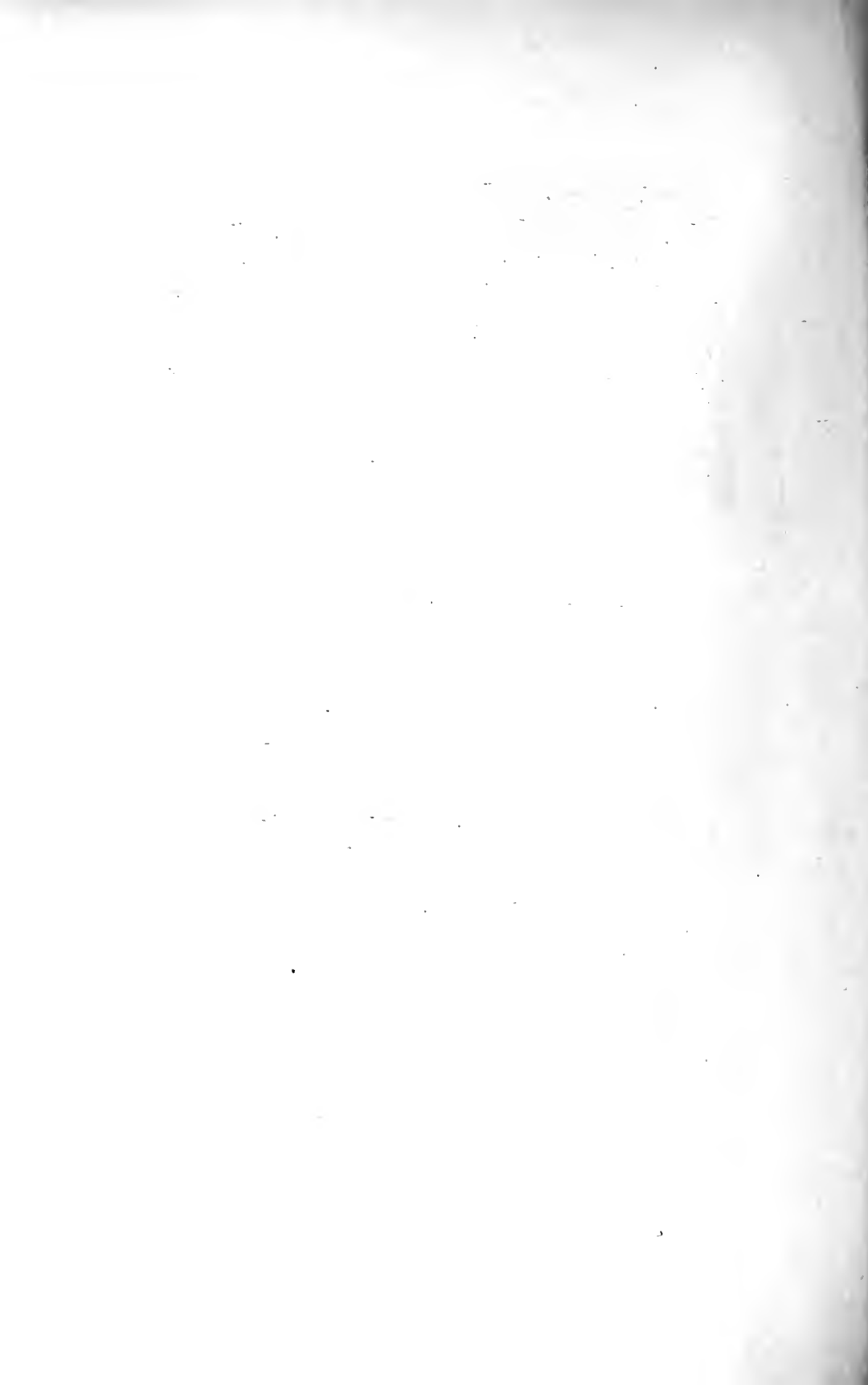


FIG. 5.

(Stewart and Rogoff: Spontaneous liberation of epinephrin.)





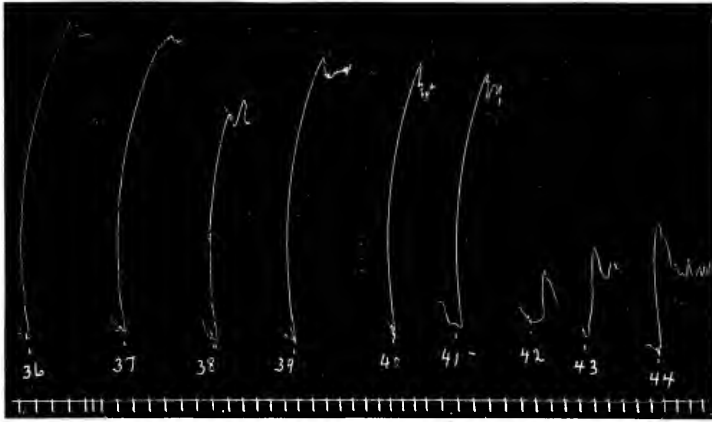


FIG. 6.

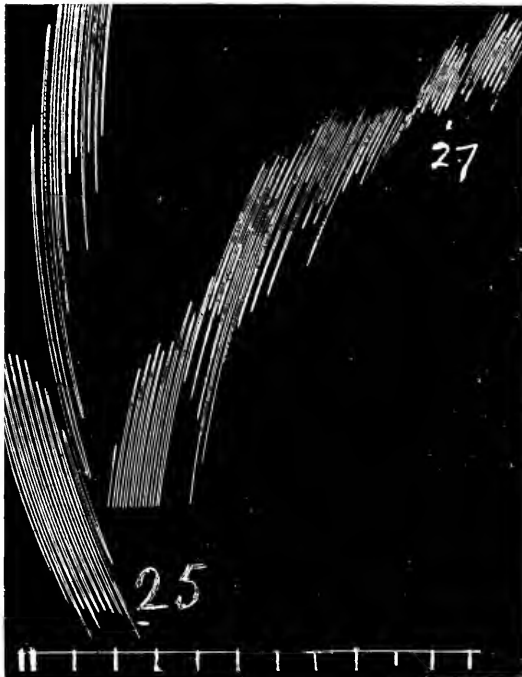


FIG. 7.

(Stewart and Rogoff: Spontaneous liberation of epinephrin.)



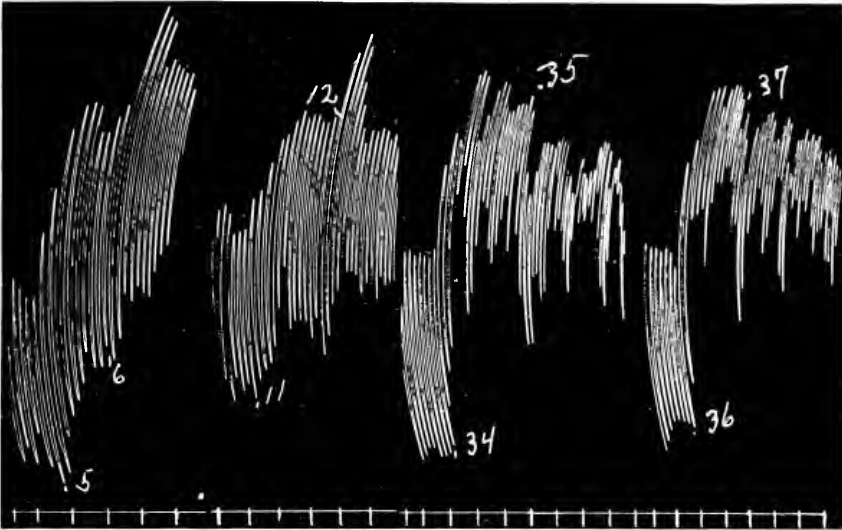


FIG. 8.

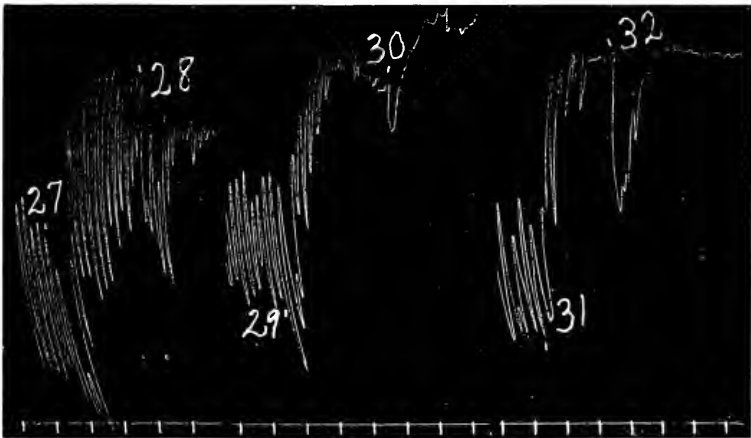


FIG. 9.

(Stewart and Rogoff: Spontaneous liberation of epinephrin.)



# EFFECT OF STIMULATION OF SENSORY NERVES UPON THE RATE OF LIBERATION OF EPINEPHRIN FROM THE ADRENALS.

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PLATES 48 TO 53.

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The fact that the spontaneous liberation of epinephrin is dependent upon the integrity of certain efferent nerves running in the sympathetic system has led to attempts to influence the rate of liberation reflexly. We have previously<sup>1</sup> published experiments in which the rates of liberation of epinephrin during and without stimulation of brachial nerves (in the cat) were compared by means of the denervated eye reactions of Meltzer and by the rise of blood pressure produced when adrenal blood collected for a known time in a cava pocket is released. The results were negative.

We have since repeated the experiments on cats and dogs, drawing off blood from the cava pocket and testing it on rabbit intestine and uterus segments. In this way the adrenal blood can, of course, be applied to the test-objects without dilution if so desired, whereas, with the eye and blood pressure reactions, it is necessarily much diluted. On the other hand, the use of the latter reactions has some great advantages. The blood is not withdrawn from the vessels, and accordingly there is no danger of loss of a part of the epinephrin in the necessary manipulations before the blood is applied to the segments. The development of the pressor property of clotted blood, a serious complication for some of the methods of testing extravascular blood (frog perfusion, artery rings), is avoided. Possible effects upon the rate of liberation of the loss of blood, or even of the loss of the epinephrin in it when withdrawn from the body are also excluded. Most impor-

<sup>1</sup> Stewart, G. N., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1916. viii, 517.

tant of all, the rise of blood pressure, especially if interpreted by the aid of the eye reactions, affords a direct quantitative comparison of the amount of epinephrin liberated in successive observations.

*Technique.*—The adrenal blood was collected through a boiled and oiled cannula in the inferior cava. The cava pocket was usually much shorter than that employed for the eye and blood pressure reactions, where it was essential to have as roomy a pocket as possible. With the short pocket only a small dead space is left filled with blood which has passed through the adrenals during one set of experimental conditions at the moment when the experimental conditions have been changed. To reduce still further any overlapping of the blood samples successively collected with and without stimulation, excitation of the sensory nerve was started slightly before completion of the collection of the preceding "no stimulation" sample, and *vice versa*. Once begun, the flow of blood from the cannula was interrupted, sample after sample being collected. The time of collection and the weight or volume of blood being accurately measured, the rate of blood flow during the collection of each sample is known. This is indispensable, of course, for estimating the rate of liberation of epinephrin by reactions which, like the segment tests, give only the concentration. Since, as we have found,<sup>2</sup> the concentration in successive samples tends to increase as the blood flow slackens, we varied the order of stimulation and no stimulation observations, interposing for example, a stimulation period between two no stimulations, and *vice versa*. Richards and Wood,<sup>3</sup> in their work on the influence of stimulation of the depressor upon suprarenal secretion, have recognized the necessity of measuring the rate of flow of the blood, and the advisability of not taking the samples in a uniform order. The stock adrenalin used for the epinephrin assays was always freshly assayed by the colorimetric method of Folin, Cannon, and Denis.<sup>4</sup>

In our previous observations we stimulated the brachial nerves, as it was convenient in forming the cava pocket to clamp the abdominal aorta, and the sciatic nerve was therefore not available. Since the upper thoracic portion of the spinal cord can sustain a substantial liberation of epinephrin after section of the cord in the cervical region,<sup>5</sup> the sciatic might perhaps be considered more likely to yield positive effects than the brachial. Elliott's conclusion<sup>6</sup> that after section of the brain stem just in front of the anterior corpora quadrigemina exhaustion of the epinephrin store of the adrenal by stimulation of sensory nerves occurs, while it does not take place if the cord has been cut just below the bulbar vasomotor center, does not, as already pointed out,<sup>1</sup> necessarily indicate that the center concerned in epinephrin liberation, on which Elliott made no experiments, is as

<sup>2</sup> Stewart and Rogoff, *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 77.

<sup>3</sup> Richards, A. N., and Wood, W. G., *Am. J. Physiol.*, 1915-16, xxxix, 54.

<sup>4</sup> Folin, O., Cannon, W. B., and Denis, W., *J. Biol. Chem.*, 1912-13, xiii, 477.

<sup>5</sup> Stewart and Rogoff, *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 143.

<sup>6</sup> Elliott, T. R., *J. Physiol.*, 1912, xlv, 374.

high as this. But if it were, brachial stimulation ought to hit it, as well as sciatic stimulation. To be sure, however, of stimulating afferent nerves favorably situated with reference to one or the other of the centers concerned, if there is more than one, we used both sciatic and brachial in these experiments.

While cats proved suitable for the previous observations in which blood was not withdrawn, it was judged advisable in the present series to use dogs also, in order to make certain of a good and uninterrupted flow of blood, which would completely wash out the pocket and cannula, and thus prevent overlapping of the successive samples.

*Experiment 1. Condensed Protocol.*—Dog; weight 10 kilos. Ether anesthesia. 45 cc. of blood withdrawn from jugular vein. Cava pocket tied off. Intestinal arteries and abdominal aorta not tied. Right iliac artery ligated and cannula inserted into the right iliac vein. The left iliac vein was not tied, and was clamped just before the collection of blood was begun, in order to spare the left sciatic as much as possible. The left sciatic and brachial nerves were prepared for stimulation. The following blood specimens were collected from the cannula in the iliac.

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.	Stimulation.
	cc.		cc.	
1	13.0	1 min., 40 sec.	8.0	None.
2	17.3	2 " 25 "	7.2	Brachial.
3	9.2	1 " 40 "	5.8	"
4	7.4	1 " 47 "	4.4	None.
5	10.2	3 " 10 "	3.2	Sciatic.
6	7.5	3 " 10 "	2.4	"
7	5.0	3 " 24 "	1.5	None.

While the pocket was still clipped off, 53 cc. of arterial blood were obtained from the carotid artery. Combined weight of adrenals 1.050 gm.

It will be seen from Figs. 1 to 3, that no difference could be made out between the inhibitory effects produced on the rabbit intestine segments by the various adrenal blood samples, which could be connected with the presence or absence of nerve stimulation. Thus in Fig. 1, the second sample collected during brachial stimulation (Observation 5) caused practically the same effect as the first sample, collected without stimulation, and the blood flows were about the same during collection of the two samples. The third sample, with nerve stimulation, gave a greater inhibition (Observation 7) than the first, without stimulation; but stimulation of the nerves had nothing

to do with this, since the nerves were also stimulated during collection of the second sample (Observation 5). The explanation of the greater inhibition produced by the third sample is that the blood flow was slower during its collection, and the rate of liberation of epinephrin per minute remaining the same, the concentration was necessarily greater. The adrenalin assays showed that the concentration in the first and second samples was somewhat more than 1:3,300,000, corresponding to an output of epinephrin per minute of 0.0025 mg. (0.00025 mg. per kilo of body weight per minute), and that the third specimen was not twice as strong as the first or second. This is a normal output for a dog under the experimental conditions, as estimated in drawn adrenal blood on rabbit intestine segments.

In Fig. 2 it is shown that sciatic stimulation also produced no demonstrable effect, the slight preponderance of the fifth sample (Observation 15) as compared with the fourth (Observation 13) being plainly associated with the somewhat slower flow during collection of the latter. In Fig. 3 the sixth and seventh adrenal samples were compared with greater magnification; but the result was the same; the blood collected during stimulation of the sciatic caused no greater effect than that collected without stimulation. It is probable, however, that the concentration of epinephrin in the sixth and seventh samples was such as to produce the maximum inhibition of which the segment was capable at the time. That the sixth specimen really contained somewhat less epinephrin than the seventh, corresponding to the greater blood flow, is brought out by the uterus tests (Fig. 4, Observations 48 and 50). The same progressive increase in concentration in the successive samples shown by the intestine tracings is exhibited on the uterus tracings, only, as is commonly the case, even more sharply. The difference between the fourth and third specimens (Observations 44 and 45) would unquestionably have been brought out clearly with a greater degree of dilution, as the increase of uterus tone in these observations was already approaching, if it had not indeed reached the maximum for the segment.

*Experiment 2. Condensed Protocol.*—Cat; weight 2.9 kilos. Urethane anesthesia. Obtained specimen of jugular blood, then isolated left sciatic nerve and prepared it for stimulation. Cava pocket made, tying off intestinal and renal vessels and right iliac artery. Cannula inserted into the cava, making a short pocket. Adrenal blood was then collected as follows:



No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.	Stimulation.
	gm.		gm.	
1	1.5	1 min., 25 sec.	1.1	None.
2	4.4	4 "	1.1	Sciatic.
3	1.4	6 "	0.23	None.
4	1.8	9 " 30 "	0.19	"
5	2.3	13 "	0.18	Sciatic.
6	1.7	8 "	0.21	None.
7	2.2	10 "	0.22	"

During stimulation of the sciatic, the pupils dilated widely, the respiratory movements were increased, and there were reflex movements indicating that the stimulus was effective. Stimulation was begun  $\frac{1}{2}$  to 1 minute before collection of the corresponding specimen, and was stopped  $\frac{1}{2}$  to 1 minute before the collection of the specimen ceased, to allow for washing out of the dead space. The flow was notably diminished after the first period of strong stimulation; that is, after the collection of the second specimen. After the last adrenal sample was collected indifferent blood was obtained from the abdominal aorta. Combined weight of the adrenals 0.347 gm.

Specimens of the tracings with rabbit segments are given in Figs. 5 to 7. They show the same general result as in Experiment 1; that is, a progressive increase in epinephrin concentration in successive adrenal samples, unmodified within the limits of sensitiveness of the method by stimulation of the sciatic.

*Experiment 3. Condensed Protocol.*—Cat; weight 2.58 kilos. Urethane anesthesia. Cava pocket prepared with cannula in left renal vein. Renal and intestinal arteries tied, but not the abdominal aorta. Sciatic nerve prepared for stimulation. Adrenal blood samples were collected as follows:

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.	Stimulation.
	cc.		cc.	
1	10.0	5 min.	2.0	None.
2	9.0	6 " 30 sec.	1.5–2.0*	Sciatic.
3	10.3	5 "	2.0	None.
4	10.0	—	—†	During asphyxia.

\* Part of the specimen was lost by accidentally spilling from the container.

† The blood clotted in the cannula at the end of the collection, and was gradually slowing so that the flow per minute could not be properly calculated.

Indifferent blood was obtained from the abdominal aorta. Indifferent blood from the carotid of another cat was also used in the tests after testing it against the blood from the abdominal aorta, and finding it to have the same tone-increasing power. Combined weight of the adrenals 0.328 gm.

The same results were obtained with the intestine and uterus segments as in the preceding experiments; there was equality or a progressive increase of epinephrin in the successive adrenal blood samples according to whether the blood flow remained constant or slackened. The output of epinephrin per minute was not modified, within the limits of accuracy of the assays, by stimulation of the sciatic. The concentration of epinephrin in the second and third adrenal specimens was about the same (1:3,500,000) corresponding to a liberation of 0.0006 mg. per minute (more than 0.0002 mg. per kilo of body weight per minute), a normal output as estimated on drawn adrenal blood by rabbit segments. The uterus segments could detect a concentration of 1:24,000,000 adrenalin in indifferent blood as compared with the indifferent blood itself.

*Experiment 4. Condensed Protocol.*—Dog; weight 8.5 kilos. Urethane and ether anesthesia. Cava pocket prepared, tying off the renal and intestinal arteries. The abdominal aorta was not clamped till just before the collection of blood. Cannula in right iliac vein. Left sciatic prepared for stimulation. About 5 cc. of blood were first collected from the pocket and discarded so as to wash the pocket free of any epinephrin which might have been liberated in the manipulations. The following adrenal samples were then collected.

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.	Stimulation.
	cc.		cc.	
1	34.0	2 min., 30 sec.	13.6	Sciatic.
2	24.0	3 "	8.0	None.
3	22.0	3 "	7.3	"
4	25.0	4 "	6.2	Sciatic.

Serum was obtained from the bloods by centrifugalization. The corpuscle sediment was about one-third of the total volume.

In this experiment serum was used for the segment tests instead of blood, so as to increase the chance of detecting any difference due to stimulation, since serum contains a greater concentration of epi-

nephren than the blood from which it is derived.<sup>7</sup> The result was the same as in the other experiments. Thus, the second adrenal specimen, collected without stimulation (Observation 15, Fig. 8) had a somewhat greater inhibitory effect upon the intestine segments than the first specimen collected during sciatic stimulation (Observation 11), corresponding to the difference in blood flow. The effect of the third specimen (Observation 6, Fig. 9) is greater than that of the second (Observation 4), and less than that of the fourth specimen (Observation 9), without apparent relation to the presence or absence of nerve stimulation. The adrenalin assay showed that the second specimen contained about 1:3,500,000 epinephrin, corresponding to 1:5,000,000 for the blood, an output per minute of 0.0016 mg. (0.0002 mg. per kilo of body weight per minute), a normal output for a dog, as estimated in this way. As this is a moderate concentration, and the first specimen contained still less, the failure of nerve stimulation to increase the concentration could not have been due to the initial concentration being near the possible maximum. The serum of the third specimen contained 1:3,000,000 epinephrin, corresponding to 1:4,300,000 for the blood. This gives the same output as for the second specimen (0.0017 mg. per minute), the concentrations being inversely proportional to the blood flows. The uterus tracings, some of which are reproduced in Fig. 10, confirm the conclusion that the first adrenal specimen (Observations 31 and 33) contained a smaller concentration of epinephrin than the second (Observations 32 and 34), although the sciatic had been stimulated during the collection of the first. Observation 30, Fig. 10, shows a much smaller effect with indifferent serum, compared with any of the adrenal sera, thus confirming the conclusion that the substance inhibiting the intestine was epinephrin.

The objection might be made that under the influence of the experimental conditions (anesthesia, trauma, etc.), the rate of liberation of epinephrin might be already so great that it could not be augmented by stimulation of afferent nerves. This objection has already been partly met by the fact that with moderate concentrations of epinephrin, as shown by adrenalin assays, nerve stimulation fails to

<sup>7</sup> Stewart and Rogoff, *J. Pharm. and Exp. Therap.*, 1916-17, ix, 393.

increase the concentration. There is no evidence that anesthetics increase the liberation demonstrably. In cats, some days after section of the spinal cord in the cervical region, we have found<sup>4</sup> that blood collected from the adrenal veins through a cannula in the inferior cava contains concentrations of epinephrin within the ordinary range, despite the fact that on account of the complete anesthesia below the level of the cord section it was not necessary to give an anesthetic. Elliott's result, that anesthetics cause diminution of the epinephrin store of the adrenals, is no proof, even if we admit that the diminution is really due in some direct way to the anesthetic, that the output of epinephrin is increased under their influence; since a diminution in the rate of formation of epinephrin would equally be accompanied by a diminution in the store.

Nevertheless, we made a number of experiments in which the animal was rendered insensitive by destruction of the cerebral cortex, or by increase of intracranial pressure without the use of anesthetics, except for a few minutes while the brain was being destroyed or the skull trephined for the insertion of the pressure bag.

*Experiment 5. Condensed Protocol.*—Dog; weight 7.6 kilos. Animal rendered insensible by destruction of the cerebral cortex with much of the underlying centrum ovale. It was shown at autopsy that none of the brain tissue behind the anterior edge of the anterior corpora quadrigemina had been destroyed. Ether was given only during destruction of the brain. Indifferent blood was obtained from the jugular vein. Cava pocket made. Right iliac artery, and intestinal and renal arteries tied off. Left sciatic nerve prepared for stimulation. Adrenal blood specimens collected as follows:

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.	Stimulation.
	<i>gm.</i>		<i>gm.</i>	
1	3.5	1 min.	3.5	None.
2	7.0	3 "	2.3	"
3	7.7	4 " 30 sec.	1.7	Sciatic.

A fourth adrenal specimen was obtained without stimulation, but clotting in the cannula prevented accurate measurement of the time (3 gm. in 4 to 5 minutes). Capacity of the cava pocket, which was a long pocket, 0.9 to 1.0 gm. of blood.

In this animal no evidence was forthcoming, any more than in the others, that stimulation of the sciatic was associated with a detectable increase in the rate of epinephrin output. A few of the tracings are reproduced in Fig. 11. The second adrenal specimen, collected without stimulation (Observation 23), caused about the same amount of inhibition of the intestine as the third specimen, collected during sciatic stimulation (Observation 25). If anything, the effect of the second was somewhat greater than that of the third specimen. The flows were not very different for the two specimens. The fourth adrenal specimen (Observation 19), collected without sciatic stimulation, produced a decidedly greater effect than the third, corresponding to the much slower flow. Here is an instance where a nerve stimulation period between two periods without stimulation ought to have shown some change of concentration, as compared either with the preceding or the succeeding period, had the nerve stimulation been capable of evoking such a change. The first adrenal specimen, without nerve stimulation (Observation 21), has a greater inhibitory effect than either the second or third. But this is doubtless due to epinephrin liberated during manipulations while the pocket was being tied off. Although it is often possible to complete the operation without any evidence of manipulative discharge, yet, in order to be sure of avoiding errors due to this cause, the first specimen was always considered suspect, if it gave a higher concentration than the second, and in that case rejected. It was, in fact, collected separately for the purpose of insuring that the succeeding specimens should not contain any epinephrin liberated by massage, etc., during the formation of the pocket.

Uterus tracings confirmed the conclusion that the second and third adrenal specimens (Experiment 5) had about the same concentration of epinephrin, and that the fourth had a greater concentration than either the second or third. Adrenalin assays showed that the concentration in the third specimen was about 1: 3,000,000, corresponding to an output of 0.0006 mg. of epinephrin per minute (about 0.0001 mg. per kilo of body weight per minute). This is rather below than above the average output in anesthetized dogs, as estimated by rabbit segments.

Elliott has stated that brain injuries such as destruction of the cerebral hemispheres cause discharge of the epinephrin store of the adrenals. Although for the reason already mentioned, this would not of itself be sufficient proof that the rate of liberation of epinephrin is sensibly increased by the brain irritation, an experiment was made in which the animal was rendered insensitive by increasing the intracranial pressure, without any brain mutilation, by a thin rubber bag introduced through a trephine hole.

*Experiment 6. Condensed Protocol.*—Dog; weight 9.4 kilos. Under ether anesthesia the skull was trephined, and a rubber bag inserted. The bag was connected with a mercury manometer. The pressure in it was increased to 250 mm. of mercury, and the ether discontinued. Artificial respiration was started, although the dog was still breathing well spontaneously. A short cava pocket was made. The abdominal aorta was tied off below the renals, and the renal vessels were tied. Brachial nerves on one side prepared for stimulation. As the blood pressure fell the intracranial pressure was diminished. Adrenal blood was collected as follows: The first specimen (5 to 6 gm.) was rejected to avoid any epinephrin liberated during manipulation.

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.	Stimulation.
	<i>gm.</i>		<i>gm.</i>	
2	7.2	45 sec.	9.0	None.
3	12.0	1 min., 35 "	8.0	Brachial.
4	12.5	2 " 20 "	5.4	None.
5	9.2	2 " 30 "	3.7	Brachial.

Combined weight of adrenals 1.20 gm.

In Fig. 12 are reproduced a few of the intestine segment tracings from Experiment 6. They show that the third adrenal specimen, collected during brachial stimulation (Observation 31), while causing a somewhat greater inhibition than the second specimen, collected without stimulation (Observation 29), does not produce as great an inhibitory effect as the fourth specimen, collected without stimulation. The fifth adrenal specimen, collected during brachial stimulation (Observation 35), causes an inhibition not conspicuously different from that caused by the fourth specimen. As before, the progressive increase in epinephrin concentration associated with the gradual de-

cline in the rate of blood flow has not been sensibly altered by the nerve stimulation. This result was confirmed by the uterus tracings, some of which are reproduced in Fig. 13. If the third adrenal specimen (Observation 52) were compared merely with the second (Observation 51), it might be thought that the greater effect on the uterus produced by the third specimen indicated an increased output of epinephrin due to stimulation of the nerve. This conclusion is at once seen to be erroneous when we compare the effect of the fourth specimen (Observation 54) with that of the third, for the fourth is as much stronger than the third as the third is stronger than the second, and the nerves were not being stimulated during collection of the fourth specimen. To show the uniformity of the tracings, Observation 52 on the third specimen was interposed between two observations (51 and 53) on the second. In Observations 56 to 59, the second to fifth specimens were compared in a greater dilution, and just as in the case of the intestine tracings no such preponderance of effect was revealed in the third and fifth specimens as would be expected if the nerve stimulation during their collection had sensibly increased the output of epinephrin.

Adrenalin assays were made on the intestine, and in this case also on the uterus segments. The intestine segments are more generally useful than the uterus for assaying the concentration of epinephrin, although for bringing out qualitative differences the uterus is frequently much more sensitive than the intestine. But with favorable uterus segments good quantitative results are also obtained. The uterus from adult or nearly adult virgin rabbits is the best in our experience for all such work. The assays indicated that the second adrenal specimen contained about 1:9,000,000 epinephrin (Fig. 13, Observations 61, 64, 65, and 66); and the third specimen more than 1:9,000,000 but much less than 1:6,000,000 (Fig. 13, Observation 68), probably not far from 1:8,000,000. The fourth specimen had a greater concentration than 1:8,000,000, though distinctly less than 1:5,000,000, and somewhat less than 1:6,000,000, probably about 1:6,500,000. If the blood flows in the protocol are compared with these concentrations, it will be seen that the concentrations are approximately in the inverse ratio of the flows. In other words, during the collection of these adrenal samples, the output of epinephrin per minute (0.001 mg. or more

than 0.0001 mg. per kilo of body weight per minute) remained approximately constant, and was not sensibly modified by stimulation of the brachial nerve.

It did not seem probable that if negative results were yielded by unmixed adrenal vein blood, positive results would be obtainable with cava blood collected by a catheter anterior to the adrenal veins.<sup>8</sup> However, as the necessary operation for the catheter method is less severe than for the cava pocket method, the possibility could not be overlooked that the output of epinephrin in the periods without nerve stimulation might be less when extensive trauma was avoided. If this were so, an increase in the liberation produced by the stimulation of nerves might more readily make itself felt. Of course, it is impossible by this method to take account of any changes in the rate of blood flow through the adrenals; and comparisons of the concentration of epinephrin in the blood are only valid for the estimation of changes in the rate of liberation if alterations in the rate of blood flow are known, unless the assumption can be made that the blood flow remains constant during the whole experimental period. Nevertheless, the conditions which in the abdominal operation lead to notable alterations in the rate of blood flow were not so likely to be present with the catheter method, except in so far as the catheter itself might interfere with the flow of blood in the cava, and therefore we made a number of experiments in this way. Experiment 7 is an example.

*Experiment 7. Condensed Protocol.*—Cat; weight 2.875 kilos.

10.00 a.m. 5 gm. urethane by stomach tube.

11.00 a.m. Exposed left femoral vein and prepared it for catheter; prepared right sciatic nerve for stimulation.

11.20 a.m. Blood I obtained through catheter inserted to a level just anterior to adrenal veins. At autopsy, the eye of the catheter was found to be about 5 to 6 mm. anterior to the orifice of the right adrenal vein when inserted to the distance used in the experiment.

11.32 a.m. Started stimulation of sciatic.

11.37 a.m. Blood II collected through catheter at the same level, sciatic stimulation being continued throughout the collection.

11.40 a.m. Blood III obtained from lower cava through catheter, which was withdrawn 9 cm.

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<sup>8</sup> Cannon, W. B., and Hoskins, R. G., *Am. J. Physiol.*, 1911-12, xxix, 274.



12.00 m. Blood IV obtained from catheter in the same manner as Blood I.

12.05 p.m. Blood V obtained from catheter anterior to the adrenal veins, collected during asphyxia.

12.09 p.m. Blood VI obtained from catheter lower down in cava, as for blood III.

After obtaining each of the bloods I, III, and IV, the catheter was withdrawn, cleaned, and oiled again. The bloods were withdrawn as uniformly as possible by the aid of an aspirator.

The result was negative. No difference was found by rabbit intestine and uterus segment tests between blood withdrawn without stimulation of the sciatic, and blood withdrawn during stimulation. None of the samples caused any inhibition of the intestine segments. We do not doubt that under favorable conditions (especially sensitive segments, slow blood flow in the inferior cava, and possibly a fortunate location of the eye of the catheter with reference to the adrenal vein orifices) samples of blood may sometimes be drawn from the inferior cava containing a sufficient concentration of epinephrin to yield distinct reactions. We failed to obtain such reactions because the epinephrin given off by the adrenals in the usual amount was too highly diluted by the cava blood. To illustrate the effect of this dilution we made some experiments, in which pure adrenal blood from the cava pocket and catheter blood from above the level of the adrenals, obtained from the same animal, were compared. In some observations catheter blood from the level of the adrenals collected while the adrenal veins were clipped was compared with blood from the same level collected through the catheter with the adrenal veins open, also with a negative result. Experiment 8 is an example of these experiments.

*Experiment 8. Condensed Protocol.*—Cat; weight 2.68 kilos. Urethane 3 gm. by stomach tube, and later on, 2 gm. more. Both adrenal veins isolated and prepared for clipping. Femoral vein prepared for catheter insertion. Blood I, drawn through catheter from level just anterior to adrenal veins; blood II, from catheter at the same level, but with adrenal veins clipped; blood III, obtained in same manner as blood I; blood IV (10 cc. in 11 minutes) obtained from cava pocket through cannula in right renal vein. After releasing the pocket by removing the clamps, blood V was obtained through the catheter in the same way as blood I. Indifferent (arterial) blood was obtained from the abdominal aorta. The catheter was withdrawn, cleaned, and oiled after each specimen was collected.

In Fig. 14 it is seen that catheter blood from the level of the adrenals displacing arterial blood (Observation 10) produced no inhibition of the intestine segment, although pure adrenal vein blood (Observation 8) caused good inhibition. Adrenalin assays showed that the concentration of epinephrin in the adrenal blood was not far from 1:3,000,000. It was confirmed on uterus segments (Fig. 15, Observations 33, 35, and 45) that the catheter blood caused no greater effect than indifferent blood, and much less than adrenal blood (Observations 34 and 36). The uterus segment, as it happened, gave practically no increase of tone with indifferent blood, which rendered the demonstration of the absence of detectable epinephrin in the catheter blood all the more convincing. Catheter blood collected with clipped adrenal veins (Observation 37) behaved in no respect differently from blood similarly collected, but with the adrenal veins open (Observation 39). The segment could easily detect epinephrin in the concentration of 1:65,000,000 (Observation 46). From the relatively considerable increase of tone given by blood with this concentration of adrenalin, there is no doubt that a much smaller concentration could have been detected. Accordingly, the adrenal vein blood must have been diluted in the inferior cava much more than twenty times.

In Fig. 16 (Observation 20) catheter blood from the level of the adrenals, collected with the adrenal veins clipped, was displaced by catheter blood collected with the veins open. No change in the intestine segment curve was produced; that is, the adrenal blood was so much diluted in the cava that a sample of cava blood containing the adrenal contribution could not be discriminated by this intestinal segment from a sample of cava blood which could not have been mixed with any adrenal blood. Nor could it be discriminated from the indifferent (arterial) blood, since its replacement by this (Observation 21) left the curve unaffected. A prompt and marked inhibition was produced, however, when the arterial blood was in its turn displaced by the pure adrenal blood (Observation 22).

In the last experiment to be quoted (Experiment 9), blood was collected by a catheter at the level of the adrenals during stimulation of the sciatic and without sciatic stimulation. Pure adrenal blood was also collected from the same dog, during and without stimulation of the sciatic.

*Experiment 9. Condensed Protocol.*—Dog; weight 14.9 kilos. Ether anesthesia. A specimen of indifferent blood was collected from the jugular vein. Then the left femoral vein was prepared for insertion of the catheter, and the right sciatic nerve prepared for stimulation. The catheter was now inserted to a level just anterior to the adrenal veins. At autopsy it was verified, as in all the other experiments of this type, that the eye of the catheter was anterior to the orifices of the adrenal veins. Three samples of blood were now collected through the catheter, the first without sciatic stimulation (13.0 cc. in 1 minute and 30 seconds), the second during sciatic stimulation (13.8 cc. in 1 minute and 56 seconds), and the third during sciatic stimulation (12.6 cc. in 1 minute and 18 seconds). The catheter was now removed, washed, oiled again, and reinserted to the same level after an interval of 6 minutes; and a fourth specimen collected through the catheter without sciatic stimulation (23.5 cc. in 3 minutes and 12 seconds). A cava pocket was now made, the renal and left iliac vessels being tied, and a cannula inserted in the left iliac vein. The abdominal aorta was clamped at the bifurcation just before beginning the collection of blood from the pocket. Adrenal blood samples were obtained from the pocket as follows:

No. of adrenal specimen.	Blood collected.	Time of collection.	Blood flow per min.	Stimulation.
	cc.		cc.	
1	15.2	1 min., 50 sec.	8.4	None.
2	16.1	2 " 5 "	8.0	Sciatic.
3	18.3	2 " 32 "	7.0	"
4	20.0	2 " 30 "	8.0	None.
5	22.9	3 " 55 "	5.7	"
6	11.4	3 " 45 "	3.0	Sciatic.
7	13.1	4 " 5 "	3.2	"
8	7.1	4 "	1.8	None.

Combined weight of adrenals, 1.15 gm.

The bloods were carefully compared on intestine and uterus segments and the degree of dilution of the adrenal blood with indifferent blood, which just caused the inhibitory effect on the intestine segments to become too slight to be detected with certainty, was determined. In Fig. 17 some of the tracings are given. At 8 the fifth adrenal blood replaced indifferent blood, causing a marked inhibition of the intestine segment. The concentration of epinephrin in this adrenal sample was assayed at about 1:3,000,000, corresponding to an output of 0.002 mg. of epinephrin per minute (0.00013 mg. per kilo of body weight per minute). In the eighth specimen the concen-

tration was assayed at 1:1,100,000, about three times as great as in the fifth sample. This is approximately the inverse ratio of the blood flows, the rate of output of epinephrin being practically unchanged. At 24 blood collected with a catheter at the level of the adrenals during sciatic stimulation replaced indifferent blood. It produced no inhibition, but instead a further increase of tone. Observations 26 and 28 represent the effect of the fifth adrenal blood specimen diluted respectively with ten and with twenty volumes of indifferent blood. Inhibition of the segment can still be detected in each case. Diluted with forty volumes of indifferent blood (Observation 30) the fifth adrenal specimen produced an inhibition so slight, if any, that it could not have been detected unless perhaps by comparison with the preceding and succeeding observations. With this dilution the concentration of epinephrin in the blood was already only 1:120,000,000, and this blood was again diluted with four volumes of Ringer's solution before being applied to the segment. At 32, the adrenal blood, diluted with eighty volumes of indifferent blood, replaced the indifferent blood. No inhibition could be clearly detected any more than with the catheter blood (Observation 24).

We fail to see how it is possible to make exact experiments on the rate of liberation of epinephrin by the catheter method.

#### SUMMARY.

An attempt was made to determine whether stimulation of afferent nerves (sciatic and brachial) produced a detectable increase in the rate of liberation of epinephrin from the adrenals, as determined by testing adrenal vein blood on rabbit intestine and uterus segments. The result was negative.

#### EXPLANATION OF PLATES.

In all the tracings time is marked in half minutes.

#### PLATE 48.

FIG. 1. Intestine tracings. Blood of a dog anesthetized with ether. At 2 Ringer's solution was replaced by jugular blood, and this at 3 by the first adrenal blood specimen, collected without stimulation of nerves. At 4 Ringer's solution was replaced by jugular blood, and this at 5 by the second adrenal blood specimen,

collected during stimulation of the brachial. At 6 Ringer's solution was replaced by jugular blood, and this at 7 by the third adrenal blood specimen, collected during brachial stimulation. Reduced one-third.

FIG. 2. Intestine tracings. Blood of the same dog as in Fig. 1. At 12 Ringer's solution was replaced by jugular blood, and this at 13 by the fourth adrenal specimen, collected without stimulation of nerves. At 14 Ringer's solution was replaced by jugular blood, and this at 15 by the fifth adrenal blood specimen, collected during sciatic stimulation. At 19 Ringer's solution was replaced by jugular blood, and this at 20 by the second adrenal specimen, collected during brachial stimulation. At 21 Ringer's solution was replaced by jugular blood, and this at 22 by the first adrenal specimen, collected without stimulation of nerves. Reduced one-third.

FIG. 3. Intestine tracings. Blood of the same dog as in Figs. 1 and 2. Greater magnification. At 24 Ringer's solution was replaced by jugular blood and this at 25 by the sixth adrenal specimen, collected during sciatic stimulation. At 27 Ringer's solution was replaced by jugular blood, and this at 28 by the seventh adrenal specimen, collected without stimulation of nerves. Reduced one-third.

#### PLATE 49.

FIG. 4. Uterus tracings. Blood of the same dog as in Figs. 1 to 3. At 41 Ringer's solution was replaced by arterial blood; at 42, by the first adrenal specimen, collected without nerve stimulation; at 43, by the second adrenal specimen, collected during brachial stimulation; at 44, by the third adrenal specimen, collected during brachial stimulation; at 45, by the fourth adrenal specimen, collected without nerve stimulation. In Observations 41 to 45 the bloods were diluted with four volumes of Ringer's solution. At 47 Ringer's solution was replaced by the fifth adrenal specimen, collected during sciatic stimulation; at 48, by the sixth adrenal specimen, collected during sciatic stimulation; at 50, by the seventh adrenal specimen, collected without nerve stimulation. In Observations 47 to 50 the bloods were diluted with nine volumes of Ringer's solution. Reduced one-half.

FIG. 5. Intestine tracings. Blood from a cat anesthetized with urethane. At 4 Ringer's solution was replaced by jugular blood, and this at 5 by the second adrenal blood specimen, collected during sciatic stimulation. At 6 Ringer's solution was replaced by jugular blood, and this at 7 by the fourth adrenal specimen, collected without sciatic stimulation. The bloods were diluted with four volumes of Ringer's solution. Reduced one-third.

FIG. 6. Intestine tracings. Blood from the same cat used for Fig. 5. At 12 Ringer's solution was replaced by jugular blood, and this at 13 by the seventh adrenal specimen, collected without stimulation of the sciatic. At 14 Ringer's solution was replaced by jugular blood, and this at 15 by the fifth adrenal specimen, collected during sciatic stimulation. The bloods were diluted with two volumes of Ringer's solution. Reduced one-third.

FIG. 7. Uterus tracings. Blood of the same cat used for Figs. 5 and 6. At 28 Ringer's solution was replaced by jugular blood; at 29, by the second adrenal blood specimen, collected during sciatic stimulation; at 30, by the fourth adrenal specimen, collected without stimulation; at 31, by the fifth adrenal specimen, collected during sciatic stimulation; at 32, by the seventh adrenal specimen, collected without stimulation. All the bloods were diluted with four volumes of Ringer's solution. Reduced one-half.

PLATE 50.

FIG. 8. Intestine tracings. Sera of a dog anesthetized with urethane and ether. At 10 Ringer's solution was replaced by serum of arterial blood, and this at 11 by serum of the first adrenal specimen, collected during sciatic stimulation. At 14 Ringer's solution was replaced by serum of arterial blood, and this at 15 by serum of the second adrenal specimen, collected without sciatic stimulation. Reduced one-third.

FIG. 9. Intestine tracings. Sera of the same dog as in Fig. 8. At 3 Ringer's solution was replaced by serum of arterial blood, and this at 4 by serum of the second adrenal specimen, collected without nerve stimulation. At 5 Ringer's solution was replaced by serum of arterial blood, and this at 6 by serum of the third adrenal specimen, collected without nerve stimulation. At 8 Ringer's solution was replaced by serum of arterial blood, and this at 9 by serum of the fourth adrenal specimen, collected during sciatic stimulation. Reduced one-third.

FIG. 10. Uterus tracing. Sera of the same dog used for Figs. 8 and 9. At 30 Ringer's solution was replaced by serum of arterial blood; at 31, by serum of the first adrenal specimen, collected during sciatic stimulation; at 32, by serum of the second adrenal specimen, collected without sciatic stimulation. The serum in Observation 30 was undiluted, in Observations 31 and 32 it was diluted with an equal volume of Ringer's solution. At 33 Ringer's solution was replaced by serum of the first adrenal specimen diluted with three volumes of Ringer's solution, and at 34 by serum of the second adrenal specimen similarly diluted. Reduced one-half.

PLATE 51.

FIG. 11. Intestine tracings. Blood of a dog rendered insensitive by destruction of the cerebral cortex. At 18 Ringer's solution was replaced by jugular blood, and this at 19 by the fourth adrenal specimen, collected without nerve stimulation. At 20 Ringer's solution was replaced by jugular blood, and this at 21 by the first adrenal specimen, collected without nerve stimulation. At 22 Ringer's solution was replaced by jugular blood, and this at 23 by the second adrenal specimen, collected without nerve stimulation. At 24 Ringer's solution was replaced by jugular blood, and this at 25 by the third adrenal specimen, collected during sciatic stimulation. All the bloods were diluted with four volumes of Ringer's solution. Reduced one-half.

FIG. 12. Intestine tracings. Bloods from a dog rendered insensitive by increased intracranial pressure. At 28 Ringer's solution was replaced by indifferent (arterial) blood, and this at 29 by the second adrenal specimen, collected without brachial stimulation. At 30 Ringer's solution was replaced by arterial blood, and this at 31 by the third adrenal specimen, collected with brachial stimulation. At 32 Ringer's solution was replaced by indifferent blood, and this at 33 by the fourth adrenal specimen, without brachial stimulation. At 34 Ringer's solution was replaced by arterial blood, and this at 35 by the fifth adrenal specimen, collected with brachial stimulation. All the bloods were diluted with two volumes of Ringer's solution. Reduced one-third.

## PLATE 52.

FIG. 13. Uterus tracings. Bloods from the same dog used in Fig. 12. At 50 Ringer's solution was replaced by indifferent (arterial) blood; at 51, by the second adrenal specimen, collected without brachial stimulation; at 52, by the third adrenal specimen, collected with brachial stimulation; at 53, by the second adrenal specimen; at 54, by the fourth adrenal specimen, collected without brachial stimulation. All these bloods were diluted with two volumes of Ringer's solution. At 56 Ringer's solution was replaced by the fourth adrenal specimen; at 57, by the third adrenal specimen; at 58, by the second adrenal specimen; at 59, by the fifth adrenal specimen, collected with brachial stimulation. These bloods were diluted with four volumes of Ringer's solution. At 61 Ringer's solution was replaced by indifferent (arterial) blood made up with adrenalin to a concentration of 1:8,000,000; at 66, by indifferent blood made up with adrenalin to a concentration of 1:9,000,000; at 68, by the indifferent blood with adrenalin to a concentration of 1:6,000,000. All the adrenalin bloods were diluted with four volumes of Ringer's solution before application to the segment. At 64 Ringer's solution was replaced by the second adrenal specimen, at 65 by the third adrenal specimen, each diluted with four volumes of Ringer's solution. Reduced one-half.

FIG. 14. Intestine tracings. Blood from a cat anesthetized with urethane. At 7 Ringer's solution was replaced by indifferent (arterial) blood, and this at 8 by adrenal blood. At 9 Ringer's solution was replaced by arterial blood, and this at 10 by catheter blood collected at the level of the adrenals. Reduced one-half.

FIG. 15. Uterus tracings. Blood from the same cat used for Fig. 14. At 33 Ringer's solution was replaced by catheter blood from the adrenal level, and this at 34 by adrenal blood; at 35 Ringer's solution was replaced by catheter blood, and this at 36 by adrenal blood. At 37 Ringer's solution was replaced by catheter blood collected during the clipping of both adrenals, and this at 38 by adrenalin (1:1,000,000) in the same blood. At 39 Ringer's solution was replaced by catheter blood from the level of the adrenals, and this at 40 by adrenalin (1:3,300,000) in the same blood. At 42 Ringer's solution was replaced by

adrenalin (1:16,500,000) in catheter blood; at 43, by the same catheter blood without the addition of adrenalin; at 45, by another catheter specimen collected at the adrenal level; at 46, by adrenalin (1:65,000,000) in the catheter blood used for Observation 45.

PLATE 53.

FIG. 16. Intestine tracings. Blood from the same cat used for Figs. 14 and 15. At 19 Ringer's solution was replaced by catheter blood collected with the adrenal veins clipped, and this at 20 by catheter blood collected at the adrenal level with the adrenal veins open. At 21 this catheter blood was replaced by arterial blood, and this at 22 by adrenal blood. Reduced one-third.

FIG. 17. Intestine tracings. Bloods from a dog anesthetized with ether. At 7 Ringer's solution was replaced by indifferent blood, and this at 8 by the fifth adrenal specimen. At 23 Ringer's solution was replaced by indifferent blood, and this at 24 by catheter blood, collected at the level of the adrenals during sciatic stimulation. At 25 Ringer's solution was replaced by indifferent blood, and this at 26 by the fifth adrenal specimen, diluted with ten volumes of indifferent blood. At 27, 29, and 31 Ringer's solution was replaced by indifferent blood, and this at 28, 30, and 32 by the fifth adrenal specimen, diluted respectively with twenty, forty, and eighty volumes of indifferent blood. All the bloods were diluted with four volumes of Ringer's solution before application to the segment. Reduced one-third.



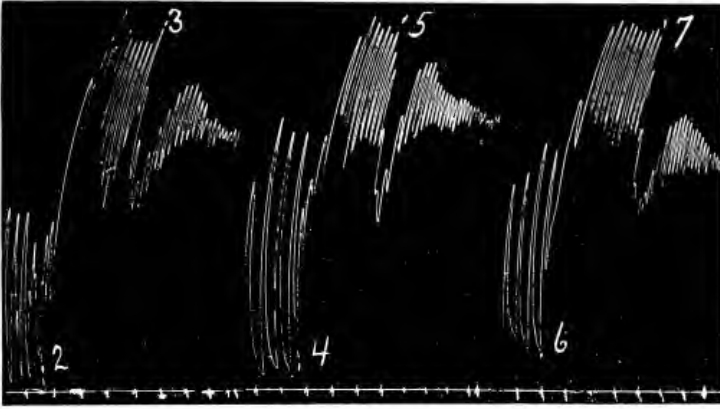


FIG. 1.

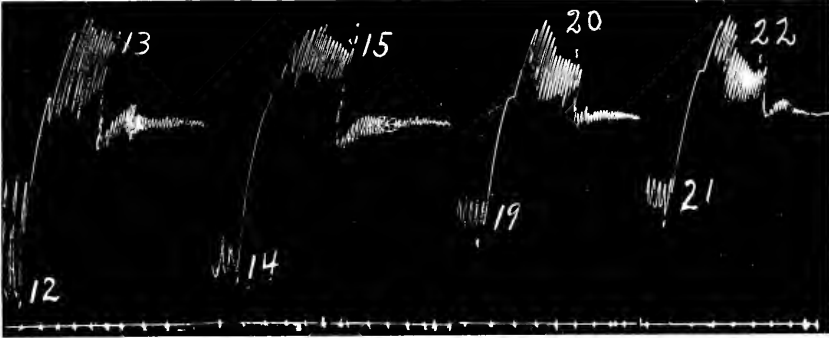


FIG. 2.

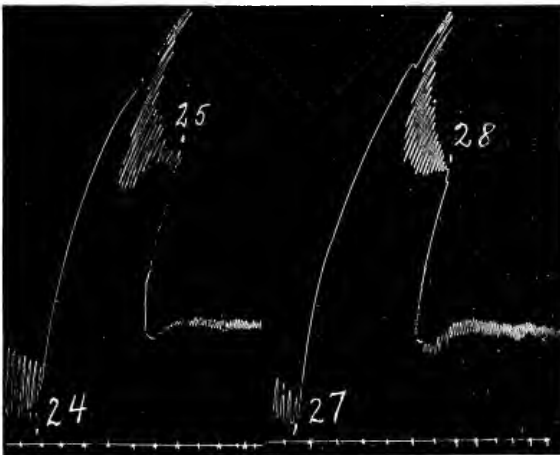
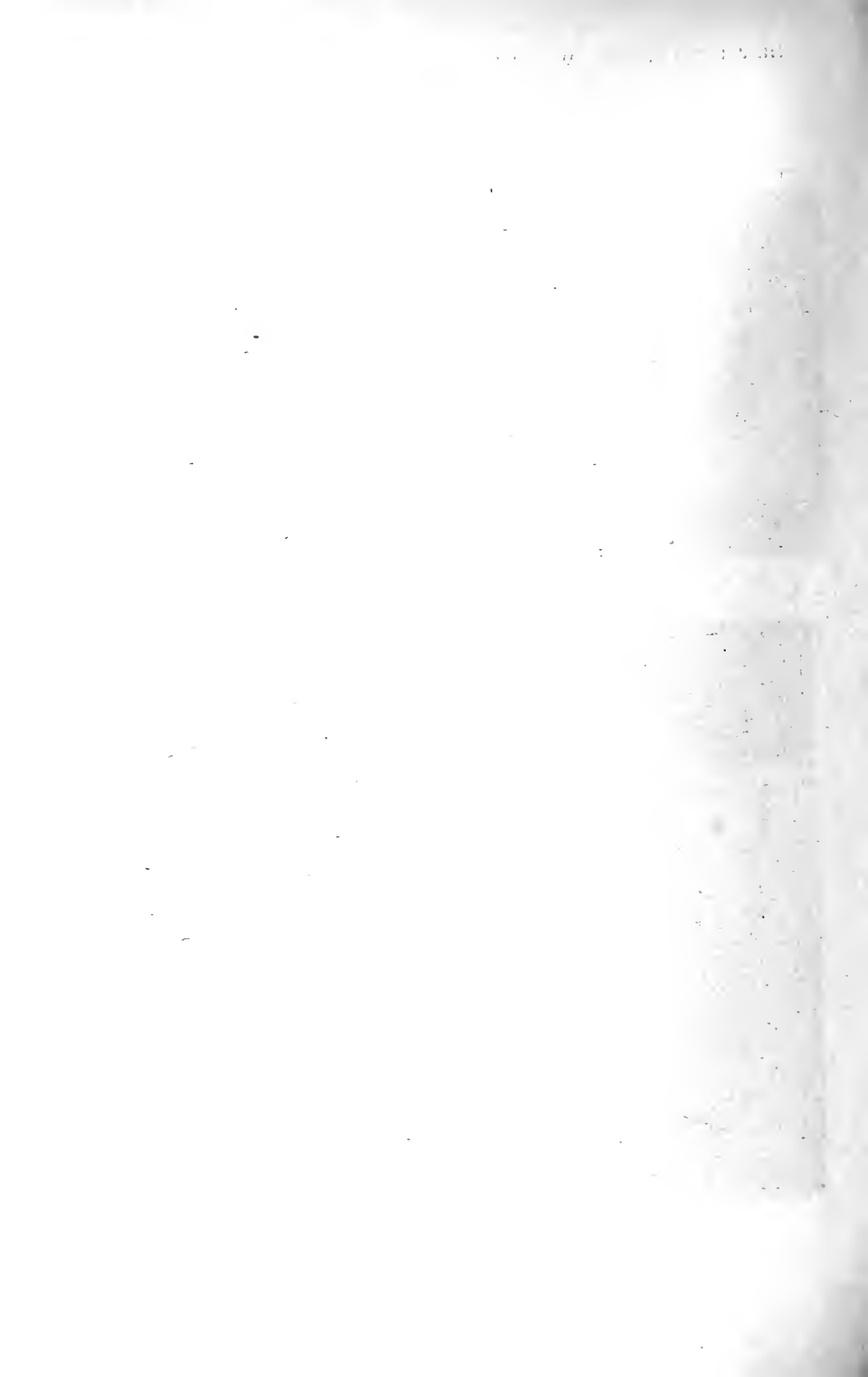


FIG. 3.

(Stewart and Rogoff: Rate of liberation of epinephrin.)



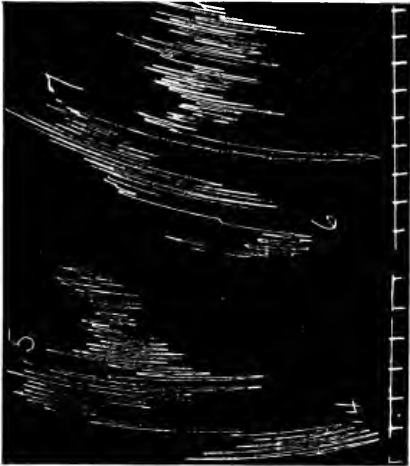


FIG. 5.



FIG. 7.

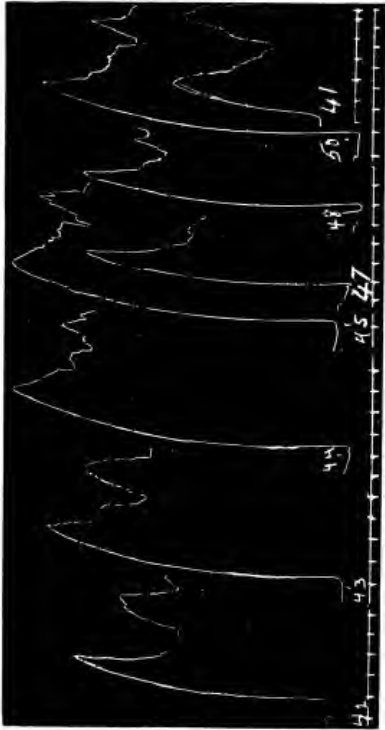


FIG. 4.

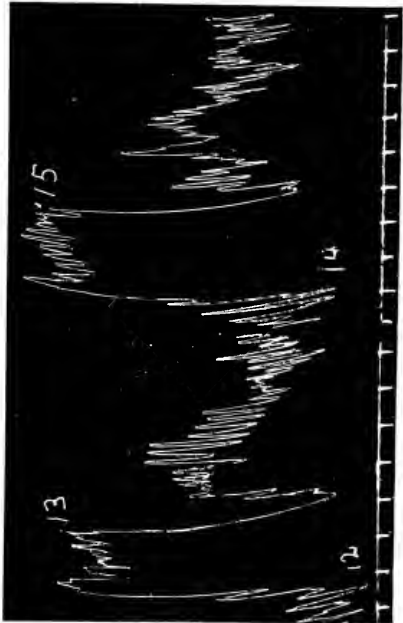


FIG. 6.



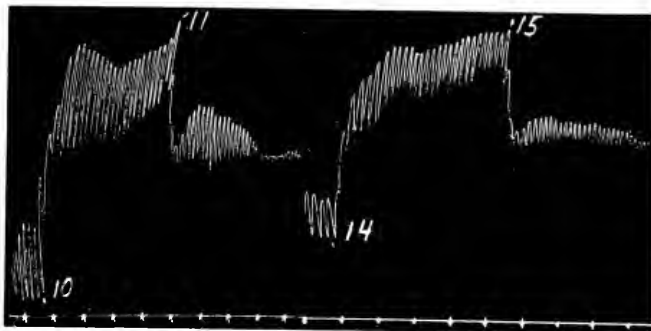


FIG. 8.

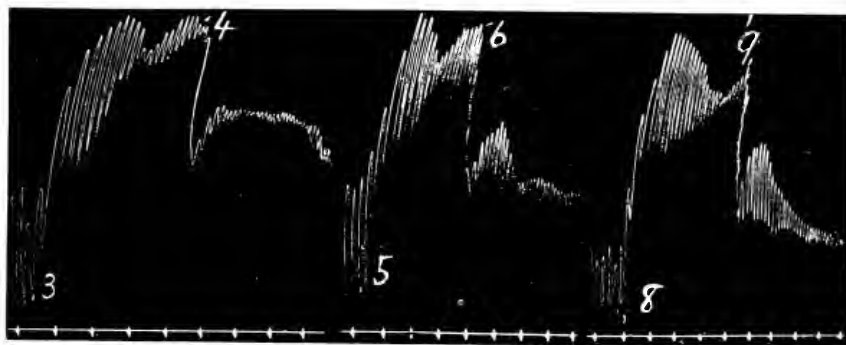


FIG. 9.

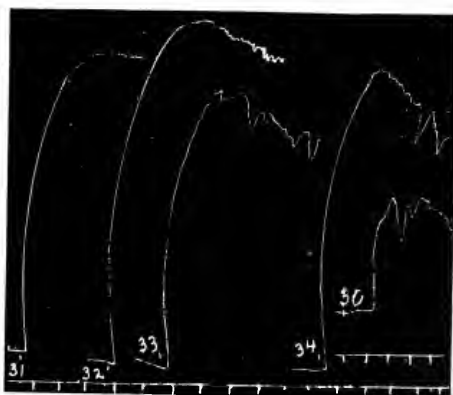


FIG. 10.

(Stewart and Rogoff: Rate of liberation of epinephrin.



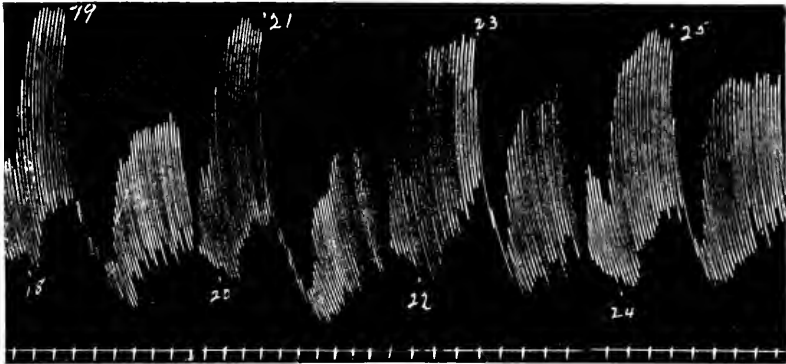


FIG. 11.

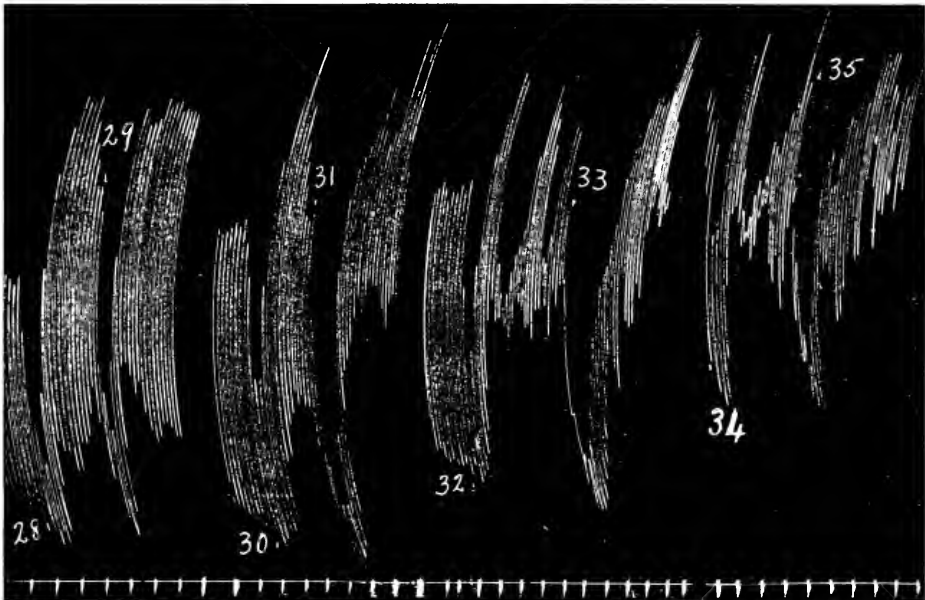


FIG. 12.

(Stewart and Rogoff: Rate of liberation of epinephrin.)





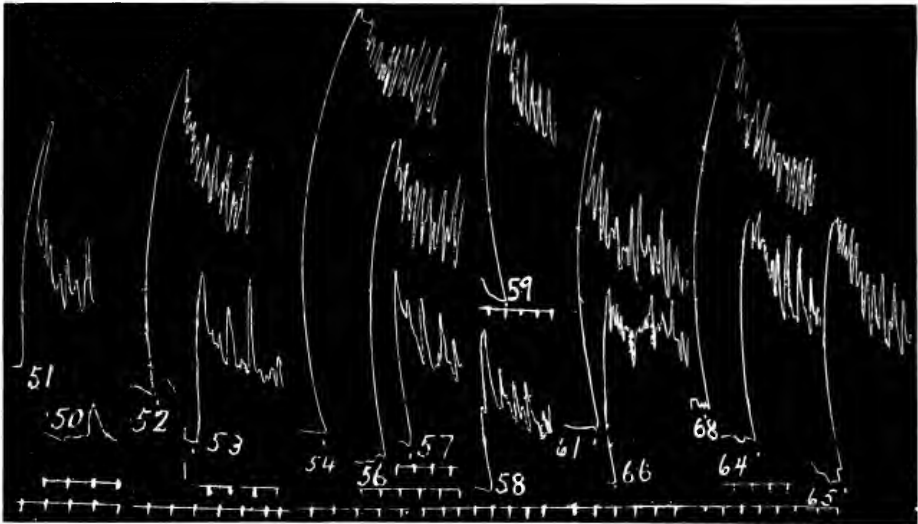


FIG. 13.

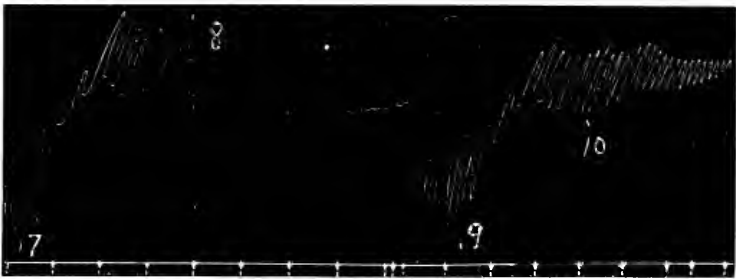


FIG. 14.

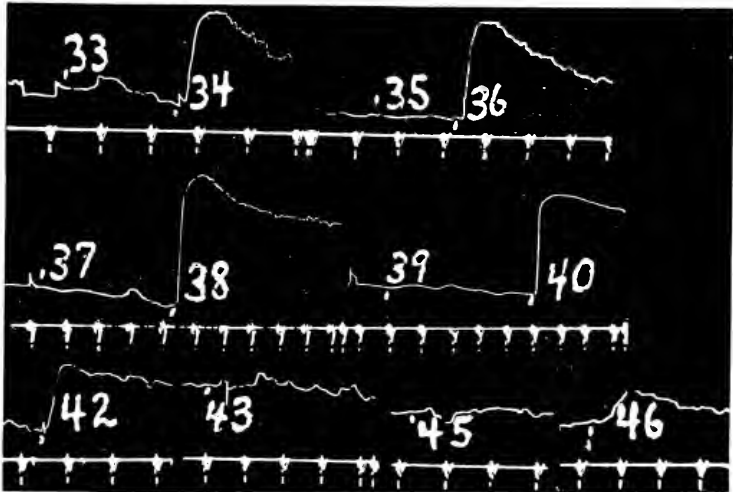
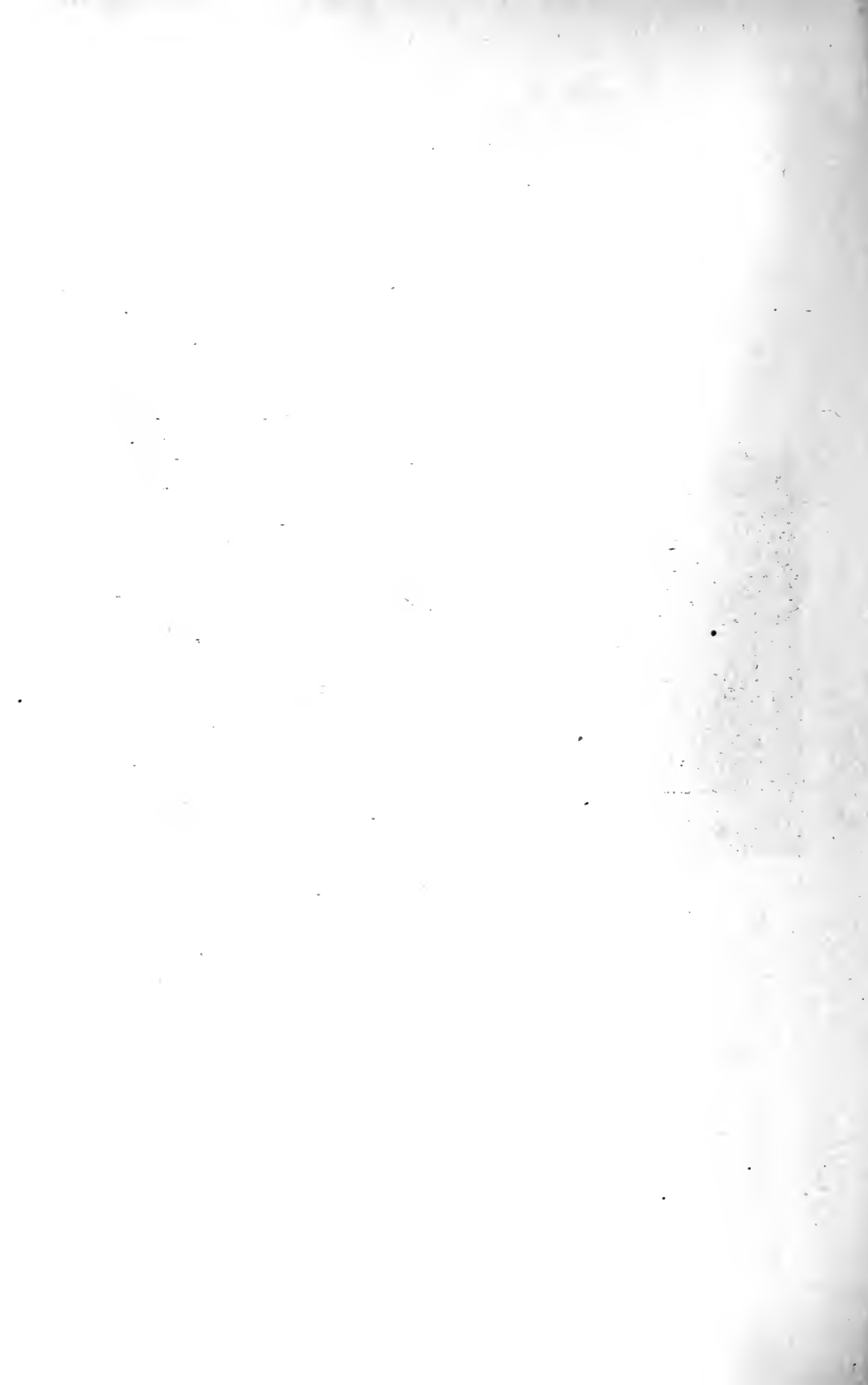


FIG. 15.

(Stewart and Rogoff: Rate of liberation of epinephrin



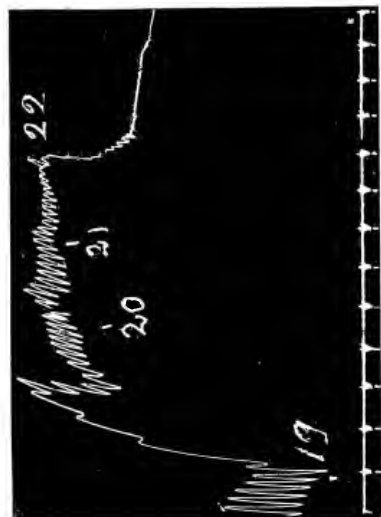


FIG. 16.

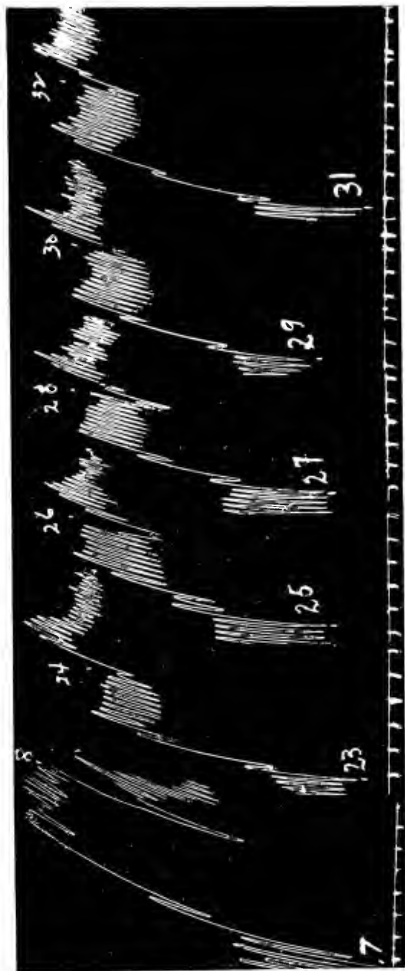


FIG. 17.

(Stewart and Rogoff: Rate of liberation of epinephrin.)



# BACTERICIDAL FLUORESCENCE EXCITED BY X-RAYS.

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(Received for publication, June 5, 1917.)

## INTRODUCTION.

The purpose of this article is to report an experiment which, coupled with underlying theoretical considerations, points to a mechanism by which the x-rays may be made to have a strong bactericidal effect.

It was suggested to me over a year ago that the fluorescence of dyes be studied from the point of view of determining whether there might be a relationship between this property and bactericidal action. The photodynamic effect of various dyes had previously been investigated, especially by Jodlbauer and von Tappeiner.<sup>1</sup> Dilute solutions of the dyes containing suspensions of various organisms were subjected to strong diffuse daylight and the results compared with controls kept in the dark. In general these experiments have given contradictory and unsatisfactory results and the influence of fluorescence as a factor was open to doubt.

Various forms of radiation have been and are being studied from the point of view of abiotic effect. Considering the spectrum as a whole from the very short or x-rays up through the ultra-violet and visible to the long infra-red and heat waves there are only two regions in which the rays have any considerable abiotic effect; namely, in the ultra-violet and infra-red. Most work has shown that the other spectral regions have very little bactericidal effect. Of these two regions the infra-red is probably not of practical value for our purposes, heat being harmful to all protoplasm. Of all radiation the ultra-violet is by far the most bactericidal and the least penetrative. Unfortunately, to be of practical value abiotic radiation must be

<sup>1</sup> Jodlbauer, A., and von Tappeiner, H., *Münch. med. Woch.*, 1904, li, 1096. Mettler, E., *Arch. Hyg.*, 1905, liii, 79.

capable of penetrating tissue. To alter the penetrative qualities of radiation is hopeless on the face of it.

From the point of view of utilization of radiant energy as a therapeutic bactericidal agent the problem therefore reduces itself to seeking to secure a transformation of penetrative energy into some form of energy having abiotic properties. Heretofore the attack has been along chemical lines. Transformation of energy is essentially a physical phenomenon and there is already at hand a very definite mechanism by which a transformation of this character may be produced; namely fluorescence. In order that this mechanism may be here applied, obviously two conditions must hold. In the first place, the fluorescent light, the light emitted by the substance when excited, should of itself have abiotic properties. In the second place, the exciting light, the light causing the fluorescence, must have considerable penetrative property. If these conditions hold, then abiotic light can be produced at any point to which the substance can be brought and the imposition of further physiological conditions would give a practical solution of the problem.

#### HISTORICAL.

The abiotic properties of radiant energy are best shown by ultra-violet light. The known ultra-violet extends from about 600 to 4000 Ångström units (0.06 $\mu$  to 0.4 $\mu$ ). Its abiotic properties have been given considerable attention, perhaps most precisely by Victor Henri and his associates.<sup>2</sup> He investigated the effect on microorganisms from about 3600 down to about 2100 Ångström units, using a few filter screens to obtain an idea as to the relative effect of different portions of the region. He concluded that the abiotic effect first became appreciable somewhere between 2800 and 3000 Ångström units and increased progressively with decrease in wave-length. Lyman<sup>3</sup> has shown that the effect increases markedly below 2000, being almost instantaneous somewhere below 1750.

We have repeated the experiments of Henri using a more precise method and have come to the same general conclusions, placing the upper limit at about 2800 Ångström units and finding an approxi-

<sup>2</sup> Cernovodeanu, P., and Henri, V., *Compt. rend. Acad.*, 1910, cl, 52, 549. Henri and Henri, *ibid.*, 1912, clv, 315.

<sup>3</sup> Lyman, T., *Spectroscopy of the extreme ultraviolet*, London and New York, 1914, 103.

mately constant effect from there to 2100. In this region the typhoid bacillus is about one two-hundredth as sensitive as the photographic plate. Below 2000 Ångström units all materials become rapidly increasingly opaque. 2 cm. of water transmits at 2000, 86 per cent; at 1930, 75 per cent; at 1860, 30 per cent; and at 1729 Ångström units, 0.5 mm. of water is opaque.<sup>4</sup> It is therefore probably safe to set the figures 1800 to 2800 as defining the useful limits of the abiotic spectrum.

While the employment of red light as a penetrating radiation offers certain ulterior advantages, physical considerations discourage the prospect of producing ultra-violet fluorescence by this means. Fluorescence is almost invariably of longer wave-length than its excitant. A good example of this is the dye fluorescein. Dilute solutions of this fluoresce a bright green in daylight but by lamplight lose the property, lamplight containing plenty of green rays but no shorter blue waves. This same general law of fluorescence renders it probable that the x-ray should produce ultra-violet fluorescence, and in fact oftener than it does visible fluorescence. The latter type of x-ray fluorescence has long been known; the discovery of x-rays was due to this property.

The fluorescence problem thus resolves itself into the discovery or manufacture of substances which shall have desirable biological properties and at the same time emit light of wave-length less than 2800 Ångström units when subjected to x-radiation. The problem is therefore one which is rational and which at the same time offers far reaching possibilities.

It is well at this point to give an outline of the work as to the effect of x-rays alone on microorganisms. The literature on this subject seems to have stopped in 1906 with the publication by Russ<sup>5</sup> of a long article which also summarizes the results of previous workers. They all, with one exception, had obtained negative results, even with very long exposures to the x-rays. A great many pathogenic organisms were tried and in the presence of various culture media. Rieder<sup>6</sup> in 1898, using an apparatus incompletely described, was able to almost sterilize agar and gelatin plates of cholera, diphtheria, typhoid, and colon organ-

<sup>4</sup> Lyman,<sup>3</sup> p. 60.

<sup>5</sup> Russ, V. K., *Arch. Hyg.*, 1906, lvi, 341.

<sup>6</sup> Rieder, H., *Münch. med. Woch.*, 1898, xlv, 101, 773.

isms, with exposure for about 1 hour. Bouillon cultures were not so clearly affected. These results are in disagreement with those of everyone else including ourselves. A study of Rieder's article, however, does not reveal any probable cause for the discrepancy. Russ performed four types of experiments. He exposed the media to determine whether exposure rendered them unfit for culture purposes. He exposed organisms under the objective of the microscope in order to observe the effect upon their motility and clumping. He exposed the organisms in the various culture media and he inoculated exposed organisms into animals. All his experiments were entirely negative except for increased motility under the microscope in some cases. His exposures were from  $\frac{1}{2}$  to 2 hours, using a fairly powerful apparatus and soft, medium, and hard tubes. Our experiments incidentally check the work of Russ.

It was shown in 1905 by Schuhknecht<sup>7</sup> that the fluorescence in fluorite excited by x-rays extends from 2310 to 3900 Ångström units with a maximum at 2840. This mineral, therefore, should be suitable to use in experiments designed to demonstrate the essential correctness of the above considerations.

We have therefore undertaken the experiments reported in the following pages. The results embody an answer to the following questions. (1) Have the x-rays any bactericidal value and if so under what conditions is it demonstrable? (2) Can the x-rays be shown to have an increased bactericidal value when the bacteria are exposed to them in contact with a substance (fluorite) of such chemical and physical constitution that the x-rays excite fluorescence in it? (3) The second question being answered in the affirmative, is the increased activity due to chemical products contributed to the medium in which the bacteria are suspended, or is it due to physical agencies, presumably rays of light in the ultra-violet region of the spectrum?

The general conditions governing the experiments were as follows. The x-ray apparatus<sup>8</sup> consists of a coarse focus Coolidge tube operated by a Snook interrupterless transformer. The tube was operated on a 4 milliamperere current backed up by a voltage equivalent to a 9 inch spark-gap. A 1 mm. aluminum filter plus black paper was used and the objects were placed at a distance of 7 inches from the anticathode. The doses were in multiples of 5 minutes, a 5 minute pause occurring between each 5 minute exposure. Fresh 18 hour agar

<sup>7</sup> Schuhknecht, P., *Ann. Physik.*, 1905, xvii, 717.

<sup>8</sup> The x-ray apparatus was kindly placed at my disposal by Dr. David R. Bowen of the Pennsylvania Hospital.



slant cultures of *Bacillus typhosus* were used, a suspension of the organisms in some medium, usually distilled water, being made. One of these suspensions stays nearly constant in count throughout the day. Each figure in the tables gives the average bacterial count per square centimeter for an agar plate made from the suspension in question.

In earlier experiments with small capillary tubes of glass as containers, certain specimens of glass gave much lower bacterial counts than the average. As glass of certain kinds is known to fluoresce under the x-rays, in order to avoid this effect paraffin was chosen as a sufficiently inert substance from which to construct containers. In order to demonstrate its inertness holes of two sizes were made, one  $\frac{1}{2}$  inch in diameter and the other  $\frac{1}{10}$  inch in diameter. These two have a considerably different ratio of wall to volume and figures obtained with them should therefore serve to demonstrate any influence of the wall. Experiments showed no difference for exposures in the two sizes of holes (Tables I and II). This at the same time serves to eliminate the size of the hole as a factor in determining the outcome of the experiment. In order to prevent evaporation from the holes they were sealed with thin sheets of paraffin.

#### *Action of X-Rays Alone.*

Table I gives the results of the exposure of water and normal salt solution suspensions of the bacteria to the x-rays.

TABLE I.

*25 Minute Exposures of Salt Solution and Water Suspensions of Bacillus typhosus.*

Medium.	Exposed.	Controls.	
		Before.	After (2 hrs.).
Salt solution, small hole.....	40, 43	180, 200	
Water, large hole.....	17, 12, 17, 17	55, 54, 60	60, 70, 70
“ small “ .....	15, 18, 17	61, 70	74
“ “ “ .....	39, 38, 41	140, 135	130
“ “ “ .....	44, 43	140, 145	

The figures are bacterial counts of a suspension of *B. typhosus* in water and normal salt solution exposed to the x-rays, the containers being  $\frac{1}{2}$  and  $\frac{1}{10}$  inch holes in paraffin blocks.

In my hands there has been uniformly a considerable reduction in the bacterial count of these suspensions. As the typhoid organism was supposed to be unaffected by the x-rays, experiments were made to determine the cause of this discrepancy. Water had never been used before as a medium. Usually the observations were made by exposing agar or gelatin plates. Even Rieder did not demonstrate an effect with bouillon as a medium. An agar plate does not make a good experiment. Presumably each individual organism after fixation in the media has already started to multiply before the necessary procedures incident to exposure are completed. As a result an agent which will kill 30 per cent of the organisms will presumably still leave in each potential colony at least one live organism and the full number of colonies will be produced. At any rate the plate experiments were tried using 25 minute exposures and protecting half the plate with  $\frac{1}{2}$  inch of lead. The experiment was entirely negative whether the plate was incubated 1 hour before radiating or exposed immediately; that is, in about 15 minutes.

Table II shows that bouillon may have a protecting effect.

TABLE II.

*25 Minute Exposures in Water and Bouillon of Bacillus typhosus.*

Medium.	Exposed.	Controls.	
		Before.	After (2 hrs.).
Bouillon culture $4\frac{1}{2}$ hrs. old. ....	900, 1,150, 1,300	750, 700	1,500, 1,700, 2,000
“ suspension, large hole. ....	50, 45, 45, 47	80, 76, 80	85, 87, 92
“ “ small “ ....	36, 25, 36	75, 60	70, 82
Water, large hole. ....	17, 12, 17, 17	55, 54, 60	60, 70, 70
“ small “ ....	15, 18, 17	61, 70	74

The figures are bacterial counts of typhoid bacilli grown in bouillon or suspended in bouillon and in water, exposure being made to the x-rays in containers consisting of  $\frac{1}{2}$  and  $\frac{1}{16}$  inch holes in paraffin blocks.

A 4 hour bouillon culture exposed 25 minutes merely had its growth during the time of exposure partially inhibited. On the contrary, a bouillon suspension made just before exposure was reduced almost one-half in count. Water suspensions under similar conditions had their bacterial counts decreased to from one-third to one-fourth.

While these experiments are not conclusive as fixing the exact action of the x-rays alone, they nevertheless demonstrate the relative constancy of an effect much less potent than that presently to be described.

It should be noted in experiments of this sort that if the number of organisms dying in successive short intervals of time during which a destructive agency is acting be plotted, the points constitute a locus which tends to have the form of a probability curve, a curve having somewhat the shape of the profile of a wide flanged bell. This is the ordinary probability relationship familiar to everyone. The organisms die rapidly during part of the time causing a heaping up of the number of deaths in a short interval. In this interval of high mortality the total number killed increases very rapidly. This period is preceded and followed by intervals in which the additions to the figures for the total dead are less rapid. If the figures giving the total dead up to any time be plotted they form a locus having a different form from the above locus for rate of death. This new locus approximates a curve whose slope (tangent of the gradient angle) at any point is given by the height of a corresponding point on the probability curve, in other words by the death rate.<sup>9</sup> It is a curve, therefore, which starts with a low slope, gradually increasing to a maximum of steepness at the point of greatest mortality, then rising less and less rapidly to approach asymptotically a horizontal repre-

<sup>9</sup> The probability curve has a form given by the equation  $y = e^{-x^2}$ . For a mathematical discussion of the subject see the article on "Probability" in the *Encyclopædia Britannica*, 9th edition. In this article Fig. 1 outlines the shape of the curve. The area of each quadrilateral would represent the number of deaths occurring in an interval of time represented by the length of its short side. The total number of deaths caused by a dose acting up to any instant of time would be represented by the sum of all the preceding quadrilaterals; that is, by the area under the curve up to that point. The values of the second half of this integral from the mid-point of the curve on, are given in the table under paragraph 9. Plotted, they give the second half of the total mortality curve, the first half of the curve being the symmetrical extension backwards of this locus. It is on a curve of this form that the figures in my tables tend to lie if they are plotted against the length of dose. The *Encyclopædia Britannica*, 11th edition, under "Probability" gives in Fig. 10 the form of the probability curve and in paragraph 99 the table of values of its integral.

senting the total number of organisms subject to death. The total number dead increases slowly at first becoming rapidly larger as the point of 50 per cent sterilization is approached. From here on the number of dead increases less and less rapidly until finally all are dead.

Slight variations in the conditions of the experiment may shift this period of rapid sterilization a little one way or the other, thus producing excessive variations in the results of experiments ending within the period. Conversely large doses will require considerable increments to produce additional deaths. The increase in energy required to kill the last 10 per cent is greater than that required to kill the middle 50 per cent.

### *Influence of Fluorite.*

The first experiments with fluorite were made with the native powder. They proved unsatisfactory on account of the controls, the powder alone killing a large percentage of the organisms. This was probably an agglutinating effect produced by shaking with the very fine microscopic crystals. Larger chipped crystals were tried but floating crystals here disturbed the uniformity of size of the drop on the plating loop. A large greenish crystal of fluorite weighing 122 gm. was then obtained from the collection of the Drexel Institute and into this a hole 1 cm. deep was drilled with a No. 42 drill (about  $\frac{1}{10}$  inch). This hole when thoroughly washed out has smooth glass-like walls and organisms suspended in water placed in it for 2 hours were uninfluenced as shown by Table III.

TABLE III.

### *Influence of Fluorite Container Alone.*

Control before.....	340, 320, 330
Fluorite hole, 2 hrs.....	360, 340, 320
Paraffin " 2 ".....	320, 330, 360
Control after.....	360, 310, 340

Water suspensions of *B. typhosus* placed in  $\frac{1}{10}$  inch holes in fluorite and paraffin and left there for 2 hours. The suspensions were then syringed out and plated.

Some difficulty was experienced in obtaining uniform loops of material for plating under the varying conditions. When plating from ordinary test-tubes the surface of the liquid is sufficiently large so that its surface tension does not influence irregularly that of the loop. With small holes or small drops the varying surface tension influences considerably the size of the drop on the loop. By placing equal quantities of material on cover-slips a fairly uniform loopful could be obtained. Suspensions placed in small holes in fluorite or paraffin were syringed out well and placed on cover-slips. The angle and velocity of the loop in leaving the drop on the cover-slip were kept about the same and the size of the drop was observed as satisfactory before plating. Control experiments were made with No. 42 holes in paraffin blocks. Table IV gives the results of exposures with the Drexel crystal.

TABLE IV.

*Bactericidal Effect of Fluorite Fluorescence.*

Exposure.	Fluorite.	Paraffin.	Control, not exposed.
<i>min.</i>			
15	2, 1, 2	80, 75, 75, 75	105, 110, 115, 111, 117
20	2, 3, 4,	70, 100, 100	160, 170
20	8, 7	140, 140	170, 170, 190

Exposures to the x-rays of water suspensions of *B. typhosus* in  $\frac{1}{16}$  inch holes in fluorite and in paraffin.

One of the 20 minute exposures giving a 95 per cent mortality (7, 8 colonies per sq. cm.) is relatively the weakest bactericidal effect obtained with this crystal in any of the experiments. The corresponding controls in paraffin showed a mortality of from 22 to 45 per cent, depending upon the length of exposure and upon ordinary experimental variations. As indicated above the 95 to 98½ per cent mortality found here means relatively more than the 50 per cent mortality of the x-rayed controls.

*Nature of the Effect.*

In order to limit the possibility of a chemical effect induced by the x-rays the procedure was adopted of placing the organisms in small

quartz capillaries and sealing with a tiny bit of paraffin at each end. This operation is entirely without effect of itself. The quartz is transparent to ultra-violet light and the arrangement limits the possible interpretations of the experiment.

The results of these exposures are given in Table V. They paralleled those made without the quartz protection. The difference between the quartz and paraffin exposures may be accidental or it may be due to some effect of the quartz itself. It is not impossible that the quartz may be slightly fluorescent under the x-rays.

TABLE V.

*The Bactericidal Effect of Fluorite Fluorescence Is Not Chemical.*

Exposures.				Controls.		
Exposure.	Fluorite and quartz.	Quartz.	Paraffin.	Stock.	Fluorite 2 hrs.	Quartz 2 hrs.
<i>min.</i>						
15	15, 14	60, 45, 40	54, 52, 48	140, 145, 130		130, 140, 130, 160
25	0, 0	40, 34	40, 43	180, 200	170, 180	
25	1, 1, 1	15, 19, 16, 17, 21, 19, 16, 18	39, 38, 41, 61	140, 135	120, 240	120, 160

Exposures to x-rays of water suspensions of *B. typhosus* enclosed in quartz tubes and placed in a hole in the fluorite crystal.

Table VI gives the results of 15 minute exposures of other crystals, with protection.

These crystals are all average specimens as seen in mineralogy collections, not being very perfect in form. They included clear white, green, and purple specimens. All were about equally active except a rather deeply colored light green crystal from Deming, New Mexico. Their visible fluorescence under the x-rays varied, being green, blue, and violet. Their weights ranged from 14 to 122 gm.

Table VII gives the results of an exposure of certain of these crystals, the suspensions being placed in the holes without quartz protection.

TABLE VI.

*Variations in Crystals, Quartz Protection.*

Origin.	Exposures.							Controls.	
	Fluorite crystals.						Quartz.	Paraffin.	Quartz.
	Drexel.	Wear, England.	Deming, N. M.	Freiberg, Saxony.	Freiberg, Saxony.	Macomb, N. Y.			
Weight in gm.	122	97	37	14	52	46			
Color.	Green.	Bluish.	Green.	White.	Purple.	Slightly green.			
Fluorescence.	Green.	Blue.	Green.	Violet.	Violet.	Green.			
Bacterial count.	15, 14	16, 15	28, 35	4, 5	8, 7	8	60, 45, 40	54, 52, 48	140, 130, 145, 130

Exposures to x-rays of water suspensions of *B. typhosus* in  $\frac{1}{16}$  inch holes in various crystals, the suspensions being protected by quartz.

TABLE VII.

*Variations in Crystals, without Quartz Protection.*

Exposure.	Crystals.				Paraffin.	Controls.
	Drexel.	Freiberg (white).	Macomb, N. Y.	Deming, N. M.		Stock.
15 min.....	2, 1, 2	1, 1, 1	2, 2, 2	10, 10, 8	80, 85, 75, 75	105, 110, 115, 111, 117
None.....	180, 180	140, 180	150, 160, 150	140, 160		180, 160

Exposures to the x-rays of water suspensions of *B. typhosus* in holes in the various crystals, together with 2 hour controls, without exposure.

The rather high mortality in fluorite shown here is probably not significant of anything but ordinary experimental variations.

There seems to be no insurmountable obstacle to the development of substances having this property of fluorite and at the same time being biologically more appropriate. It is hoped that a new and productive field of experimental therapy has been opened up.

## CONCLUSIONS.

X-ray fluorescence has been pointed out as a mechanism offering exceptional possibilities in the development of physicochemical therapy.

Experiments are given which demonstrate that under these conditions the x-rays may have a strong bactericidal effect.

The x-rays alone have a partial bactericidal action on water suspensions of typhoid bacilli.

I take pleasure in expressing my indebtedness to Dr. Paul A. Lewis for his advice in the development of these conceptions and the carrying out of the experiments.



## HEMOLYSINS OF VEGETABLE ORIGIN.

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(Received for publication, December 20, 1916.)

In previous communications I reported the results of an investigation on the bacterial agglutinins, precipitins (bacterial and albuminous) and hemagglutinating substances found in the sap of *Cotyledon scheideckeri*. The present paper deals with the hemolysins which I have discovered in the sap of this plant.

2 cc. of sap in a 1:10 dilution were mixed with 0.5 cc. of a 10 per cent suspension of sheep erythrocytes in isotonic salt solution (0.85 per cent) and were left for  $\frac{1}{2}$  hour at 37°C. The mixture was then thoroughly shaken, whether agglutination was observed or not, and was again left at 37°C. for  $\frac{1}{2}$  hour. The result is given in Table I.

TABLE I.

No. of plant.	Date of collecting leaves.	Hemagglutination.	Hemolysis.*
1	Jan. 7	+	Strong.
2	" 20	—	None.
3	" 20	—	"
4	" 20	+	Strong.
5	" 7	+	"
6	" 20	+	Present.
7	" 20	—	None.
8	" 20	+	Strong.
9	Feb. 4	+	Present.
10	" 4	+	Strong.
11	" 4	+	"
12	" 4	+	Slight.

\* The three degrees of hemolysis correspond to the intensity of coloration due to hemoglobin: strong, present, and slight.

The experiments show (Table I) that hemolysis is observed only in sap that contains hemagglutinins; when the *Cotyledon* sap is deprived of hemagglutinins it does not possess hemolysins. Moreover, complete

hemolysis never appears in *Cotyledon* saps, and the hemolytic power is measured by the intensity of color of the liquid covering the erythrocytes. According to von Liebermann, ricin also never gives complete hemolysis. The experiment can be performed with ordinary test-tubes, but I used centrifuge tubes as they permit a more rapid procedure, and I submitted the suspension to centrifuging after  $\frac{1}{2}$  hour at  $37^{\circ}$ .

It has been stated that the agglutinated erythrocytes were thoroughly shaken in *Cotyledon* sap; and the agglutinated clot again furnished a homogeneous suspension, at least macroscopically homogeneous. This restoration of the original suspension is necessary in order to obtain hemolysis, as is shown in the following experiment.

2 cc. of sap in a 1:10 dilution were mixed with 0.5 cc. of a 10 per cent suspension of sheep erythrocytes and left at  $37^{\circ}$  for  $\frac{1}{2}$  hour. After  $\frac{1}{2}$  hour the tubes with the saps containing hemagglutinins were left at  $37^{\circ}$  for 2 hours, without shaking the agglutinated erythrocytes (Table II).

TABLE II.

No. of plant.	Date of collecting leaves.	Hemolysis.
4	Jan. 20	None.
9	Feb. 4	"
10	" 4	"
11	" 4	"
12	" 4	"

The comparison of these results with the corresponding results in Table I proves that we cannot obtain hemolysis without thoroughly shaking the agglutinated erythrocytes.

Ehrlich and Baumgarten, cited by Fraenkel, observed similar phenomena with ricin. Von Liebermann, in his study of hemolysis in ricin, also shook the agglutinated corpuscles. Fraenkel does not consider this absolutely necessary, having observed that ricin in large amounts is able to dissolve red corpuscles directly.

Ehrlich calls attention to the fact that agglutination considerably reduced the superficies of the erythrocytes accessible to the action of the surrounding fluid and thus prevents hemolysis in the sap.

I have previously shown that the bacterial agglutinins, precipitins, and hemagglutinins of *Cotyledon* can be bound by corresponding antigens. This is also the case with the hemolysins of *Cotyledon scheideckeri*.

2 cc. of sap in a 1:10 dilution were mixed with 0.5 cc. of a 10 per cent suspension of sheep corpuscles and left at 37° for  $\frac{1}{2}$  hour; the mixture was then centrifuged, the sap was decanted, and the inner surface of the tube and the erythrocytes were carefully washed three times with isotonic salt solution in order to remove the remaining vegetable sap. 2 cc. of isotonic salt solution were introduced into the tube, the erythrocytes were thoroughly shaken, and the mixture was left at 37° for  $\frac{1}{2}$  hour.

TABLE III.

No. of plant.	Date of collecting leaves.	Hemolysis.
4	Jan. 20	Strong.
	Feb. 4	"
7	Jan. 20	"
9	Feb. 4	Present.
13	" 4	Slight.
10	" 4	Strong.
11	" 4	"
12	" 4	Slight.

The results (Table III) show that hemolysins of vegetable sap can be bound with erythrocytes; after fixation the hemolysis can take place not only in the sap of plants, but also in isotonic salt solution. In the latter case the hemolytic power remains the same (Table IV); neither in the sap nor in the isotonic salt solution do we obtain complete hemolysis, though further observations still showed that hemolysins were present in the vegetable sap after the binding of a part of the hemolysins by a suspension of erythrocytes (Table VI).

TABLE IV.

No. of plant.	Date of collecting leaves.	Hemolysis.	
		In vegetable sap.	In isotonic salt solution.
14	Mar. 6	Strong.	Strong.
15	" 6	"	"
16	" 6	"	"
17	" 6	"	"

Moreover, a comparison of Tables III and V shows that the hemolytic power of the red cells which had bound the *Cotyledon* hemolysin does not increase even when they are introduced into fresh sap containing the original quantity of hemolysins.

2 cc. of sap in a 1:10 dilution were mixed with 0.5 cc. of a 10 per cent suspension of sheep corpuscles (0.05 cc. of sediment) and left at 37° for  $\frac{1}{2}$  hour; the mixture was centrifuged, the sap decanted, the corpuscles and the inner surface of the tube were washed three times with isotonic salt solution; 2 cc. of fresh vegetable sap of the same plant were introduced into the tube, the erythrocytes were thoroughly shaken, and the mixture was left again at 37° for  $\frac{1}{2}$  hour.

TABLE V.

No. of plant.	Date of collecting leaves.	Hemolysis.
4	Jan. 20	Strong.
	Feb. 4	"
9	" 4	Present.
10	" 4	Strong.
12	" 4	Slight.

The last two experiments led to the conclusion that either a definite quantity of red cells is able to bind only a quantity of hemolysins which is insufficient for complete hemolysis, or that the red cells are so modified during the process of binding the hemolysins by other substances contained in the sap and also by the red cells themselves that they can no longer be completely hemolyzed.

I shall return to this question after describing a series of related experiments. It seemed important to determine whether the sap possessed free hemolysins after being incubated for  $\frac{1}{2}$  hour at 37° with the 0.5 cc. of a 10 per cent suspension of erythrocytes remaining in the sap; in other words, how much hemolysin, measured by the amount of hemolyzed erythrocytes, was contained in the sap of each plant?

2 cc. of sap in a 1:10 dilution were mixed with 0.5 cc. of a 10 per cent suspension of sheep corpuscles, left for  $\frac{1}{2}$  hour at 37°, and then centrifuged; the corpuscles recovered from the sap were washed three times with isotonic salt solution, and tested for hemolysis for  $\frac{1}{2}$  hour at 37° in 2 cc. of salt solution; the decanted sap was again mixed with fresh erythrocytes (0.5 cc. of a 10 per cent suspension) and

left in the thermostat for  $\frac{1}{2}$  hour. This was repeated as long as the erythrocytes extracted hemolysin from the vegetable sap, and consequently were undergoing hemolysis in 2 cc. of isotonic salt solution (Table VI).

TABLE VI.

No. of plant.	Date of collecting leaves.	Hemolysis. 0.5 cc. of erythrocytes.								Total quantity of erythrocytes hemolyzed.
										cc.
4	Feb. 4	Strong.	Strong.	Strong.	Strong.	Strong.	Strong.	Strong.	None.	3.5
9	" 4	Present.	Present.	Slight.	None.	None.	None.	None.	"	1.5
10	" 4	Strong.	"	None.	"	"	"	"	"	1.0
11	" 4	"	Strong.	"	"	"	"	"	"	1.0
12	" 4	"	"	Slight.	"	"	"	"	"	1.5

The experiments of Table VI show that 0.5 cc. of a 10 per cent suspension of sheep corpuscles is unable to bind the total amount of hemolysin contained in the sap.

Moreover, the amount of hemolysins found in the sap of different plants is subject to the same fluctuations as that of bacterial agglutinins, precipitins, and hemagglutinins (Table VI).

Fraenkel and von Liebermann showed that the hemolytic power of ricin increased in proportion to the quantity of this phytalbumin. These authors disagree only as to the question of the limit of this power; while Fraenkel succeeded in obtaining complete hemolysis, von Liebermann never observed it.

TABLE VII.

No. of plant.	Date of collecting leaves.	Hemolysis.	
		10 per cent suspension.	5 per cent suspension.
8	Feb. 25	Strong.	Strong.
18	" 25	"	"
19	" 25	Present.	Present.
20	" 25	Strong.	Strong.
21	Mar. 6	"	"

The experiments described above prove that the presence of superfluous hemolysins in *Cotyledon* sap has no influence upon the intensity of hemolysis and never produces the total dissolution of the red cells. Table VII gives the experiments undertaken in order to observe the hemolytic processes in undissolved *Cotyledon* sap, of course possessing far greater quantities of hemolysins than the 1:10 dilutions used for the preceding experiments.

The procedure was the same as in the experiments given in Table I, except that a 5 per cent suspension of sheep red cells was used in addition to the 10 per cent suspension. Complete hemolysis was not observed.

Like the bacterial agglutinins, precipitins, and hemagglutinins already described, the *Cotyledon* hemolysins possess a strong avidity for red cells (Table VIII) and cannot be separated from them after 1 hour either at 45° or 75°.

0.5 cc. of a 10 per cent suspension of sheep corpuscles was mixed with 2 cc. of sap in a 1:10 dilution and left at 37° for  $\frac{1}{2}$  hour; the sap was decanted, the erythrocytes and the inner surface of the tube were washed three times with isotonic salt solution, then 3 cc. of isotonic salt solution were added to the corpuscles, and the liquid was shaken and centrifuged. 1 cc. of salt solution taken after centrifuging, and consequently colored from hemolysis, did not produce hemolysis with 0.5 cc. of a 5 per cent suspension of fresh erythrocytes. It was superfluous, therefore, to wash the red cells again, which had bound the hemolysin. The cells left in the tube in 2 cc. of salt solution were shaken and heated for 1 hour at 45° and 75°, then rapidly centrifuged in the heated tubes; the salt solution was decanted and its hemolytic power was tried against fresh sheep cells, as in the experiments of Table I.

TABLE VIII.

No. of plant.	Date of collecting leaves.	Hemolysis.	
		45°.	75°.
8	Feb. 25	None.	None.
18	" 25	"	"
19	" 25	"	"
20	" 25	"	"

Table IX shows that hemolysis can be obtained not only at 37° but also at room temperature after binding the hemolysins with the erythrocytes.

TABLE IX.

No. of plant.	Date of collecting leaves.	Hemolysis.
4	Jan. 20	Strong.
7	" 20	"
9	Feb. 4	Present.
10	" 4	Strong.
11	" 4	"
12	" 4	Slight.

It has already been mentioned in connection with Table VI that saps of different plants possess different quantities of hemolysins, and this is true of all other antibodies. The same method was applied to the experiments of Table X which had been used to determine

TABLE X.

No. of plant.	Date of collecting leaves.	Solution of sap.	Sap in 2 cc. of solution.	Hemolysis.	Hemagglutination.
4*	Feb. 4	1 : 10	cc.	Strong.	+
		1 : 50	0.2	"	+
		1 : 100	0.04	"	+
		1 : 200	0.02	"	+
		1 : 250	0.01	"	+
		1 : 300	0.008	Slight.	+
7*	Jan. 20	1 : 10	0.006	None.	+
		1 : 50	0.2	Strong.	+
		1 : 100	0.04	"	+
		1 : 200	0.02	Present.	+
		1 : 250	0.01	Slight.	+
		1 : 300	0.008	None.	+
9*	Feb. 4	1 : 10	0.2	Present.	+
		1 : 50	0.04	Slight.	+
		1 : 100	0.02	None.	+
		1 : 200	0.01	Slight.	+
10	" 4	1 : 10	0.2	Strong.	+
		1 : 50	0.04	"	+
		1 : 100	0.02	Slight.	+
		1 : 200	0.01	None.	-
12	" 4	1 : 10	0.2	Slight.	+
		1 : 50	0.04	None.	+

\* The limit of hemagglutination is represented by Solutions 4, 1:700; 7, 1:300; and 9, 1:200.

the quantities of bacterial agglutinins, precipitins, and hemagglutinins in *Cotyledon* sap. Solutions of the sap were prepared and the final quantity of sap that no longer contained hemolysins was observed. The method was like that described in Table III.

The next problem was to determine the influence of high temperatures upon *Cotyledon* hemolysins. The sap of each plant was heated to temperatures of 90°, 100°, 120°, 134°, and 144° for 1 hour; it was then filtered, and the experiment with the heated sap was conducted in the way described in the protocol of Table III.

Table XI represents not only the influence of high temperatures upon hemolysins, but also upon hemagglutinins.

The thermostability of the hemolysins varies in different plants, like that of the other antibodies in *Cotyledon* sap.

The suppression of the hemolytic properties at a certain temperature and their reappearance at higher ones such as 134° and 144° (Nos. 18, February 25; 19, February 25 and March 6; 20, February 25 and March 6; and 15, March 6) is a phenomenon of particular interest.

A similar fact has been described by Landsteiner and von Rauchenbichler in regard to staphylococcal hemolysins which recover their activity at 100° after inactivation at 65°. Landsteiner explains this by assuming that at 65° the hemotoxin is bound with other substances contained in the filtrate of staphylococcal cultures, and at 100° the hemotoxin is again free. We may apply this explanation to the analogous phenomenon in *Cotyledon* sap.

The reappearance of the hemolytic properties at higher temperatures after apparent inactivity makes it seem possible that still higher temperatures, which for practical reasons could not be applied, would again deliver the bound hemolysins.

We must also take into consideration the fact that the sap which recovers its hemolytic power after a temporary inactivity is able to cause complete hemolysis of sheep red corpuscles, and that the quantity of the hemolysin of sap which can be bound by the erythrocytes is sufficient for a total dissolution of the latter; if, nevertheless, we do not observe complete hemolysis, we may suppose that the quantity of hemolysins in question, instead of being directly bound with the



TABLE XI.

No. of plant.	Date of collecting leaves.		Temperature.	Hemagglutination.	Hemolysis.
			°C.		
4	Feb.	4	100	+	Strong.
			110	+	"
			120	+	"
			134	+	"
			144	+	None.
7	Jan.	20	90	+	Strong.
			100*	+	None.
8	Mar.	6	90	+	Present.
			100*	+	None.
9	Feb.	4	90	+	Present.
			100*	+	None.
10	"	4	90	+	Strong.
			100*	+	None.
12	"	4	90	+	Slight.
			100*	+	None.
18	"	25	100	+	Present.
			120	+	None.
			134	+	"
			144	—	Total.
	Mar.	6	100	+	Strong.
			120	+	"
			134	+	Present.
			144	+	None.
19	Feb.	25	100	+	Slight.
			120	+	None.
			134	+	Total.
			144	+	"
	Mar.	6	100	+	Strong.
			120	+	None.
			134	—	Total.
22	"	6	144	—	"
			90	+	Strong.
			100*	+	None.
20	Feb.	25	100	+	Strong.
			120	+	None.
			134	+	"
			144	—	Total.
	Mar.	6	100	+	Strong.
			120	+	Present.
			134	+	None.
14	"	6	144	—	Total.
			90	+	Slight.
			100*	+	None.
15	"	6	100	+	Slight.
			120	+	None.
			134	—	Total.
			144	—	"

\* Even at 120, 134, and 144° there was no hemolysis. In this table some data are omitted to avoid repetition.

erythrocytes, is bound with some other substance contained in *Cotyledon* sap and bound with the erythrocytes through its second receptor, in many saps temperatures of 134° and 144° destroy this unknown substance and thus set free a part of the hemolysins; these hemolysins added to those that are bound with the erythrocytes directly are now able to cause complete hemolysis. At the same time this unknown substance, or perhaps another one destroyed at similar temperatures, modifies the erythrocytes to such an extent that it is impossible to dissolve them completely even in distilled water (Table XII). On this account we must conclude that the absence of complete hemolysis in the experiments described above is due to some substance destroyed in many saps at the temperatures of 134° and 144°, thus preventing the appearance of complete hemolysis either by binding a part of the hemolysins needed for complete hemolysis, or by a reversible alteration of the erythrocytes so as to make them inaccessible to the action of any other hemolytic agents, and of the part of hemolysins directly bound with the red cells, which perhaps would be sufficient by itself to hemolyze the red cells completely.

In order to test the hemolytic power of distilled water against sheep red corpuscles, I proceeded as in the experiments described in Table III; but distilled water was used for washing the tube and the erythrocytes, and the latter were shaken in distilled water. Both the experiment and the control were made with 0.5 cc. of a 5 per cent suspension of sheep corpuscles and 4.5 cc. of distilled water.

The experiments of Table XIII show the hemolytic power of *Cotyledon* sap against the red cells of the sheep, horse, goose, and frog. The procedure was the same as in the experiments of Table III.

Thus *Cotyledon* sap hemolyzes erythrocytes of different species of vertebrates.

TABLE XII.

No. of plant.	Date of collecting leaves.	Hemolysis.	
		5 cc. of 10 per cent suspension in (2 cc.) isotonic salt solution.	Distilled water.
8	Feb. 25	Strong.	Strong.
18	" 25	"	"
19	" 25	Present.	"
20	" 25	Strong.	"

TABLE XIII.

No. of plant.	Date of collecting leaves.	Hemolysis of erythrocytes of different species.			
		Sheep.	Horse.	Goose.	Frog.*
4	Feb. 4	Strong.	Slight.	Strong.	Present.
9	" 4	Present.	Present.	Present.	"
13	" 4	Slight.	None.	None.	None.
10	" 4	Strong.	"	Strong.	Present.
11	" 4	"	Present.	"	"
12	" 4	Slight.	Slight.	"	"

\* The hemolysis of frog erythrocytes takes place slowly and repeated shaking is necessary. The binding of hemolysin also proceeds slowly; it is better to prolong it for 1 hour instead of  $\frac{1}{2}$  hour.

The next problem was to establish the relation between hemolysins on the one hand and bacterial agglutinins, precipitins, and hemagglutinins of *Cotyledon scheideckeri* on the other.

The experiments in Tables I and II show that the vegetable sap contains hemolysins only when it contains hemagglutinins, or, according to former studies, when it possesses antibodies in general. Table XIV shows that whenever the hemagglutinins are extracted from the sap the hemolysins simultaneously disappear.

2 cc. of sap were freed of hemagglutinins by fractional addition of different quantities of a 10 per cent suspension of sheep corpuscles and subsequent centrifugation and were mixed with 0.5 cc. of a 10 per cent suspension of sheep cells; the mixture was left for 2 hours at 37°; the cells were shaken every half hour.

TABLE XIV.

No. of plant.	Date of collecting leaves.	Hemolysis.	Hemagglutination.
4	Jan. 20	None.	—
7	" 20	"	—
9	Feb. 4	"	—
10	" 4	"	—
12	" 4	"	—

The identity of hemagglutinins and hemolysins is contradicted by the fact that the activity of hemagglutinins persists during the temporary inactivity of the hemolysins (Table XII). The opposite

phenomenon, that vegetable sap retains its hemolytic properties in spite of the absence of hemagglutinins, seems to confirm the difference of these two elements, but it can be explained even without rejecting the theory of the identity of the processes of agglutination and hemolysis (held by Ehrlich, Fraenkel, von Liebermann): as to the substance preventing hemolysis which disappeared at high temperatures, we may assume that the complete hemolysis we observe is due to some substance at once able to hemolyze and to agglutinate the red cells depending on various accessory conditions.

TABLE XV.

No. of plant.	Date of collecting leaves.	Horse serum necessary to extract precipitins (1:5).	Hemolysis.	Precipitation.
23	Feb. 4	0.6	None.	None.
9	" 4	0.3	"	"
10	" 4	0.3	"	"
11	" 4	0.4	"	"
12	" 4	0.5	"	"

TABLE XVI.

No. of plant.	Date of collecting leaves.	Bacteria necessary to extract agglutinins.	Hemolysis.	Hemagglutination.
23	Feb. 4	1.0 tube.*	None.	—
9	" 4	0.7 "	"	—
10	" 4	0.5 "	"	—
11	" 4	0.5 "	"	—
12	" 4	0.6 "	"	—

\* A whole culture was washed off with 1 cc. of isotonic salt solution.

At any rate it is impossible to affirm categorically the identity of the hemolysins and the hemagglutinins of *Cotyledon scheideckeri*; we can only establish the parallelism of their presence in the sap and their simultaneous extraction by erythrocytes (Table XIV).

Likewise, the hemolysins are extracted from *Cotyledon* sap together with bacterial agglutinins (Table XVI) and precipitins (Table XV).

The quantity of horse serum or bacterial mass (*Bacillus typhi*) necessary for the extraction of all precipitins or bacterial agglutinins from 2 cc. of sap in a 1:10

dilution was first determined. The previously ascertained quantity of serum (inactivated at 56°) or of bacteria was then introduced into 2 cc. of fresh sap in a 1:10 dilution; after precipitation (30 minutes at room temperature) or agglutination (1 hour at 37°) the mixture was centrifuged, the liquid decanted, and its hemolytic properties were tested as in the experiments of Table III. An experiment was done to control the complete absence of precipitins or agglutinins.

The experiments in Table XVII show the influence of animal serum upon the hemolytic power of *Cotyledon* sap.

Horse serum inactivated for  $\frac{1}{2}$  hour at 56° was added to 2 cc. of sap in a 1:10 dilution; the mixture was shaken and 0.5 cc. of a 10 per cent suspension of sheep cells was added. After centrifuging for  $\frac{1}{2}$  hour at 37° the liquid was decanted, and the tube and the cells were washed with isotonic salt solution. The experiment was continued like those in Table III.

TABLE XVII.

No. of plant.	Date of collecting leaves.	Serum added.	Precipitation.	Hemagglutination.	Hemolysis.
		cc.			
4	Feb. 4	0.1	+	—	—*
		0.01	+	+	—
		0.002	+	+	—
9	" 4	0.01	+	+	—
10	" 4	0.01	+	+	—
11	" 4	0.01	+	+	—
12	" 4	0.01	+	+	—

\* When the experiment was conducted like those of Table I the results were the same.

The serum prevents hemolysis in vegetable sap; in these experiments the lack of parallelism between the hemolytic and hemagglutinating properties of the sap is also evident. The quantities of serum used for the experiment (0.01 to 0.002 cc.) deprived the sap of its hemolysins, but did not influence the activity of its hemagglutinins. (The lack of parallelism between hemolysis and hemagglutination in the experiments of Table X is also evident. The agglutination of erythrocytes often takes place in the absence of hemolysis.) Only large quantities of serum (0.1 cc.) destroy both these properties at once.

However, the serum prevents hemolysis only when it is introduced

into the sap before the addition of erythrocytes. In all the experiments the serum produced precipitation; on this account we may assume that the hemolysins pass into the precipitate (Table XVII).

In this experiment the serum was introduced after the red cells; hemolysis went on normally, because the red cells had time enough to bind the hemolysin before the addition of serum (Table XVIII).

0.5 cc. of a 10 per cent suspension of sheep cells was mixed with 1.9 cc. of sap; the mixture was shaken, immediately (20 to 40 seconds) afterwards serum was added, and the entire solution was shaken again. The mixture was left for  $\frac{1}{2}$  hour at 37°, and then centrifuged. The liquid was decanted, the inner surface of the tube and the red cells were washed with isotonic salt solution three times, and the experiment was continued like those of Table III.

TABLE XVIII.

No of plant.	Date of collecting leaves.	Serum added.	Hemagglutination.	Hemolysis.
		cc.		
4	Feb. 4	0.01	+	Strong.
9	" 4	0.01	+	Present.
10	" 4	0.01	+	Strong.
11	" 4	0.01	+	"
12	" 4	0.01	+	Slight.

The experiments of Table XVIII also show the short interval of time necessary for binding the hemolysins with the erythrocytes.

The experiments of Table XIX confirm the supposition that the serum deprives the *Cotyledon* sap of its hemolysins because it causes the precipitation of the hemolysins in the solution, and not because it is present in the sap.

TABLE XIX.

No. of plant.	Date of collecting leaves.	✓ Serum added.	Hemolysis.
		cc.	
9	Feb. 4	0.01	Present.
10	" 4	0.01	Strong.
11	" 4	0.01	"
12	" 4	0.01	Slight.

These experiments were conducted like those of Table III, but in this case serum was added to the mixture of erythrocytes (bound with hemolysins) and isotonic salt solution. The total quantity of liquid in the experiment was 2.5 cc. Table XIX shows that hemolysis was always observed.

A series of experiments was undertaken to determine the reciprocal relation of the vegetable hemolysins and the immune hemolytic amboceptor.

0.5 cc. of a 5 per cent suspension of sheep corpuscles was sensitized for 1 hour at 37° by 1 cc. of rabbit hemolytic amboceptor against sheep corpuscles containing three hemolytic doses. The total quantity of liquid was 2.5 cc. The mixture of erythrocytes and hemolytic serum was centrifuged, the liquid decanted, and the sensitized erythrocytes were washed three times with isotonic salt solution, mixed with 2 cc. of sap in a 1:10 dilution, and left for  $\frac{1}{2}$  hour at 37°. Hemagglutination was always observed in the saps. The liquid was again decanted, the erythrocytes were washed, and the cells shaken in 2 cc. of salt solution, and left for  $\frac{1}{2}$  hour at 37°.

TABLE XX.

No. of plant.	Date of collecting leaves.	Hemolysis.
8	Jan. 20	Strong.
9	Feb. 4	Present.
10	" 4	Strong.
11	" 4	"
12	" 4	Slight.

The presence of hemolysis in the absence of complement (Table XX) indicates the following: either the *Cotyledon* hemolysins are bound by a group of erythrocytes different from the receptor bound with the hemolytic amboceptor, or the same receptor can be bound by the vegetable hemolysin and the hemolytic amboceptor, yet the *Cotyledon* hemolysin possessing a greater avidity supplants the amboceptor.

The experiments of Table XXI confirm the second hypothesis; the erythrocytes being bound with the vegetable hemolysins, they become inaccessible to the hemolytic amboceptor; the hemolysins of vegetable sap adhere to the erythrocytes because of their greater avidity.

The experiments were conducted like those of Table III, but in this case 1 cc. of hemolytic serum containing 3 hemolytic doses was added after the erythro-

cytes were washed. The mixture was left for 1 hour at 37° and then centrifuged; the liquid was decanted and tested for the presence of the hemolytic amboceptor (1 cc. of liquid plus 0.5 cc. of a 5 per cent suspension of sheep corpuscles plus 1 cc. of guinea pig complement, left for 1 hour at 37°); the erythrocytes were carefully washed and tested as in the experiments of Table III.

TABLE XXI.

No. of plant.	Date of collecting leaves.	Hemolysis of erythrocytes bound with sap and afterward mixed with hemolytic amboceptor.	Presence of hemolytic amboceptor after centrifuging cells bound with hemolysin <i>Cotyledon</i> .
23	Feb. 4	Strong.	Total hemolysis.*
9	" 4	Present.	" "
10	" 4	Strong.	" "
11	" 4	"	" "
12	" 4	Slight.	" "

\* The control experiment proved that the quantity of hemolytic amboceptor left free after sensitizing erythrocytes not modified by sap was insignificant; in the presence of 0.1 cc. of complement it caused weak hemolysis with abundant precipitation of erythrocytes on the bottom of the tube.

Further experiments (Tables XXII and XXIII) also confirm the hypothesis that the same receptor forms the object of binding for the vegetable hemolysin as well as for the hemolytic amboceptor, and that in consequence the two hemolysins cannot be bound with the red cells simultaneously, because the hemolysin possessing a greater avidity leaves no place for the other one.

The experiments of Table XXII prove that the complement is unable to produce complete hemolysis of red cells sensitized by the hemolytic amboceptor and afterwards modified by *Cotyledon* sap.

Experiments were conducted like those of Table XX; the sensitized red corpuscles were submitted to the action of *Cotyledon* sap, carefully washed, and thoroughly mixed with 1 cc. of guinea pig complement in a dilution of 1:10. This quantity of complement during  $\frac{1}{2}$  hour caused complete hemolysis of 0.5 cc. of a 5 per cent suspension of sheep corpuscles, in the presence of three hemolytic doses of amboceptor, the total quantity of liquid in the tube being 2.5 cc. The tube was then filled with isotonic salt solution until the quantity of liquid was 2.5 cc., and was left for  $\frac{1}{2}$  hour at 37°.



TABLE XXII.

No. of plant.	Date of collecting leaves.	Hemolysis.
9	Feb. 4	Present.
10	" 4	Strong.
11	" 4	"
12	" 4	Slight.

In the present case we must attribute the absence of complete hemolysis to the disappearance of the hemolytic amboceptor from the erythrocytes and not to the incapacity of the hemolytic system to dissolve completely the erythrocytes modified by the sap. This is proved by the fact that the complement is left free in the liquid (Table XXIII).

Experiments were conducted like those of Table XXII, but in this case only 1 cc. of complement was added to the sensitized red cells altered by the vegetable sap. The tube, not filled with salt solution, was shaken and left for  $\frac{1}{2}$  hour at 37°. After centrifugation the liquid was decanted and tested in order to discover the presence of the complement (1 cc. of liquid plus 1 cc. of hemolytic serum in three doses plus 0.5 cc. of a 5 per cent suspension of erythrocytes).

TABLE XXIII.

No. of plant.	Date of collecting leaves.	Presence of complement in guinea pig serum after elimination of sensitized erythrocytes modified by <i>Cotyledon</i> .
23	Feb. 4	Total hemolysis.
9	" 4	" "
10	" 4	" "
11	" 4	" "

The hemolysins like the bacterial agglutinins, precipitins, and hem-agglutinins, were left when the sap, in a 1:10 dilution, was filtered in a Chamberland filter. These experiments were conducted with the sap of Plants 8, 18, 19, and 20, on February 25.

## CONCLUSIONS.

1. The sap of *Cotyledon scheideckeri* possesses hemolysins for the red corpuscles of different animals.

2. The hemolysins of vegetable sap can be bound by erythrocytes and cannot be separated.

3. A definite quantity of erythrocytes is able to extract from the sap only a part of the hemolysins it contains.

4. The quantity of hemolysins in the sap of different plants is subjected to the same fluctuations as that of bacterial agglutinins, precipitins, and hemagglutinins.

5. As the hemolysins are bound by erythrocytes, the hemolysis can take place not only at 37° but also at 15–16°.

6. The thermostability of the hemolysins varies from one individual plant to another.

7. In many cases the vegetable sap loses its hemolytic properties at a certain temperature and recovers them at a higher one.

8. The vegetable sap is unable to produce complete hemolysis of erythrocytes.

9. But the sap of many plants acquires the power to dissolve red corpuscles completely after 1 hour of heating at 134° and 144°.

10. Erythrocytes modified by *Cotyledon* sap cannot be dissolved completely even by distilled water.

11. The agglutination of the erythrocytes and their hemolysis are conditioned, probably, by different substances.

12. The hemolytic amboceptor and the hemolysin of *Cotyledon scheideckeri* can be bound with the same receptor of the erythrocytes.

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## STUDIES ON THE DEVELOPMENT OF TOXICITY IN INTESTINAL SECRETION.

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(Received for publication, May 25, 1917.)

The following study was conducted with the purpose of further investigating the production of toxic material in closed loops of high intestine in dogs. These experiments are a continuation of the work of Davis,<sup>1</sup> and of Stone, Bernheim, and Whipple.<sup>2</sup> In the work of Davis, intestinal secretion, collected by irrigation of the duodeno-jejunum, excluding pancreatic secretion, was kept under chloroform and toluene a number of days in a warm place, and studied for its toxic properties. These fluids were found to be toxic, and it was felt that the intestinal secretion was, possibly, toxic at the moment of its elaboration. In the light of subsequent work, however, the author feels that his conclusions are subject to revision. The condition under which the fluid was kept did not absolutely exclude the possibility of bacterial growth. Whipple, Stone, and Bernheim<sup>3</sup> studied the toxic properties of fluids collected in closed loops of intestine left *in situ*, and certain inferences were reached as to the source of the toxin in these fluids. The present article deals with a further effort to investigate these inferences, in consequence of which light has been thrown on them from a different point of view. In the work referred to, the rapidity with which toxins developed,

<sup>1</sup> Davis, D. M., Intestinal obstruction: Formation and absorption of toxin, *Bull. Johns Hopkins Hosp.*, 1914, xxv, 33.

<sup>2</sup> Stone, H. B., Bernheim, B. M., and Whipple, G. H., The experimental study of intestinal obstruction, *Ann. Surg.*, 1914, lix, 712.

<sup>3</sup> Whipple, Stone, and Bernheim, Intestinal obstruction. I. A study of a toxic substance produced in closed duodenal loops, *J. Exp. Med.*, 1913, xvii, 286; II. A study of a toxic substance produced by the mucosa of closed duodenal loops, 307.

the generally assumed paucity of bacteria in high intestinal loops, and certain other reasons, led to the assumption that the activity of the mucous membrane of closed loops was the essential factor in toxin production, and that bacteria probably played a minor part, if any, in this process. Our present data justify the conclusion that toxins indistinguishable, as far as the symptoms and anatomical lesions produced by them are concerned, from those found in closed loops, may be elaborated from intestinal secretion *in vitro*, and hence make questionable the theory of the essential part played by the living mucosa of closed loops.

*Toxicity of Intestinal Secretion Heated to 90–95°C.*

As a point of departure for this work we desired to determine whether intestinal loop secretion, obtained in such a manner that it was as nearly normal as possible, was in itself toxic. In order to do this, it was necessary not only to make an effort to exclude bacterial action, as by the addition of chloroform and toluene, but also to eliminate the action of enzymes present in the secretion. For this purpose the following experiment was devised. Under anesthesia, the intestine was divided just distal to the lower pancreatic duct, and again at a point in the high jejunum so as to isolate a loop of duodenojejunum. The upper and lower ends of this loop were brought out of the abdominal cavity, and connected with rubber tubing so that the isolated loop could be washed through and the washings collected. The loops were irrigated with distilled water. A given volume of water, usually 1 liter, was passed through the loop repeatedly so that large quantities of fluid would not have to be handled. A number of experiments were carried out with this operative technique, the loop being washed by a slow, continuous stream for periods ranging from  $3\frac{1}{2}$  to 10 hours. The washings were collected over a boiling water bath, the temperature in the collecting flask being 90–95°C. This temperature may be assumed to be destructive of both enzymes and bacteria, so that fluid so collected and reheated for  $\frac{1}{2}$  hour on the 2 succeeding days, to allow for the presence of spore-bearing organisms, remains unchanged, if kept with aseptic precautions. Fluid so collected was injected intravenously into small

dogs, in two experiments, and produced no symptoms whatever. In other experiments, conducted under anesthesia, injection of this fluid caused no change in blood pressure.

*Toxicity of Unheated Intestinal Secretion.*

It might be suspected, however, that the heating itself destroyed a toxin normally present in intestinal secretion. To eliminate this possibility, the washings were collected as described above, except that they were not heated. This fluid was immediately injected intravenously into a small dog, also without the production of any symptoms. In addition, in two other experiments, similar unheated fluids, which had been preserved for a time by the addition of chloroform and toluene, were injected after driving off these substances at a comparatively low temperature with the aid of reduced pressure, without producing symptoms. From these experiments, we concluded that the intestinal secretion, as produced in these loops is devoid of toxic properties, and that if it is heated immediately at 90–95°C. and kept sterile, the development of toxic properties is prevented.

The next step was to discover whether this non-toxic fluid would take on, outside the body, the toxic properties characteristic of closed loop contents. Unheated loop washings, collected in sterile flasks and kept as far as possible from external contamination, were incubated at 37°C. for 18 hours. The fluids at this time showed evident and profuse bacterial growth. Upon intravenous injection, in two experiments, these incubated washings caused the death, in about 6 hours, of small dogs. In both cases the symptoms and anatomical pictures were the same as those seen after the injection of closed loop contents. The small intestine was intensely injected and hemorrhagic, most markedly in the upper duodenum. In one case, the quantity used represented the collection during 40 minutes, and in the other during 80 minutes.

*Toxicity of Unheated Intestinal Secretion Treated with Chloroform and Toluene.*

The development of toxic properties by the loop washings might be ascribed to the influence of bacterial growth or to the action of

enzymes, probably proteolytic, derived from the intestinal mucosa. In either case, the substrate would have to be the proteins of the succus entericus, no other substance except distilled water being present. A method of differentiation by which either enzymic or bacterial action alone could be made responsible for the change is difficult to devise. Passage of loop washings through a Berkefeld filter was not attempted, since the adsorption of a large part or all of the enzymes by the material of the filter might be expected.

Fractional sterilization of the loop washings offered little, since growth of bacteria occurs between sterilizations, and in addition the temperature of sterilization approaches that at which enzymes are injured.

Finally experiments were carried out in which unheated loop washings were treated with chloroform and toluene. Portions, in one case one-half the amount collected in  $3\frac{1}{2}$  hours, and in one case one-third the amount collected in 4 hours, were kept several days and then freed of the preservative substances by distillation. The second portion had in addition been incubated at  $37^{\circ}\text{C}.$  for 18 hours. It was hoped that this procedure, while preventing bacterial growth, would allow opportunity for any enzymes present to act. The fluids were injected intravenously immediately after the distillation, and no symptoms of poisoning were observed in the dogs used.

The other portions of loop washings from the experiments concerned were incubated for 18 hours at  $37^{\circ}\text{C}.$ , after being freed from the preservatives by distillation at reduced pressure, and then injected. In one case in which the distillation was carried out at a temperature of about  $80^{\circ}\text{C}.$  the injection of the fluid in a test dog produced no symptoms of poisoning. In the other, a better vacuum was obtained, and the temperature of the distillation never exceeded  $60^{\circ}\text{C}.$  The injection of this fluid produced a period of shallow respirations and weak pulse during the injection, but at the end the animal was in good condition and ran about the floor before being put in the cage. Extreme prostration supervened in 4 hours and the dog died during the ensuing night. There was no diarrhea. At autopsy the intestine was pale and showed nothing abnormal except the presence of a rather large amount of gas. The content of the small intestine was a bile-stained fluid. The mucosa showed no

hemorrhage or congestion. This picture is entirely unlike that caused by the injection of closed loop fluid, and has never been observed by either of the authors in long series of such injections.

In both these last experiments, the fluids, after removal of the preservatives and incubation for 18 hours, showed growth of *Bacillus subtilis* in spite of the precautions taken. It is probable that the spores of this organism survived the treatment with chloroform and toluene.

From these experiments, we cannot say positively that bacteria are responsible for the development of toxicity in loop washings, but they offer no support to the conception that enzymes are the cause of such development. These experiments, together with the previous ones reported by one of us (Davis<sup>4</sup>), show that loop washings can become toxic outside the body, producing symptoms like those caused by closed loop fluid. In this case there can be no question of the perverted activity of the epithelial cells of the mucosa. In addition, it is clear that unless chloroform and toluene inhibit the enzymes in this intestinal fluid, their action alone does not produce the toxic properties.

#### SUMMARY.

1. Intestinal secretion, collected by the method described in this paper, is non-toxic when fresh.

2. This secretion, when heated immediately to 90–95°C. and kept sterile, remains non-toxic.

3. This secretion, when not heated, remains non-toxic when kept under chloroform and toluene, even if incubated at 37°C.

4. This secretion, when not heated, but collected in a sterile flask, becomes toxic upon incubating 18 hours, producing symptoms like those of closed loop fluid.

5. The secretion, when treated with chloroform and toluene, and later incubated for 18 hours, after these preservatives have been removed by distillation at 60°C., does not produce lesions typical of closed loop fluid.

<sup>4</sup> Davis, Natural immunity of animals against poison of intestinal obstruction, *Bull. Johns Hopkins Hosp.*, 1914, xxv, 39.





## A COMPARATIVE STUDY OF SERUM AND LYMPH FERMENTS AFTER FEEDING.

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(Received for publication, June 2, 1917.)

In a preceding paper Jobling and his associates<sup>1</sup> have discussed the ferment changes that are observed in the serum of dogs after feeding. During the course of that work certain abrupt alterations in the titer of the ferments were occasionally encountered, changes that seemed to us to appear more rapidly than would be anticipated from gradual absorption following the stimulation of the ferment-producing organs.

In order to study these relations more fully we have undertaken a comparison of the lymph and serum alterations following feeding, collecting the lymph from a thoracic duct fistula and taking samples of the blood at varying time intervals.

The technique used was the same as described in the previous paper<sup>1</sup> except that the action of the proteolytic ferments has been accelerated by incubating at from 47° to 50°C., which represents more nearly the optimum temperature than 37°C., heretofore used. The peptidase (ereptase) has been estimated by the dilution method with the digestion of a peptone solution and subsequent production of the tryptophane reaction with bromine water.

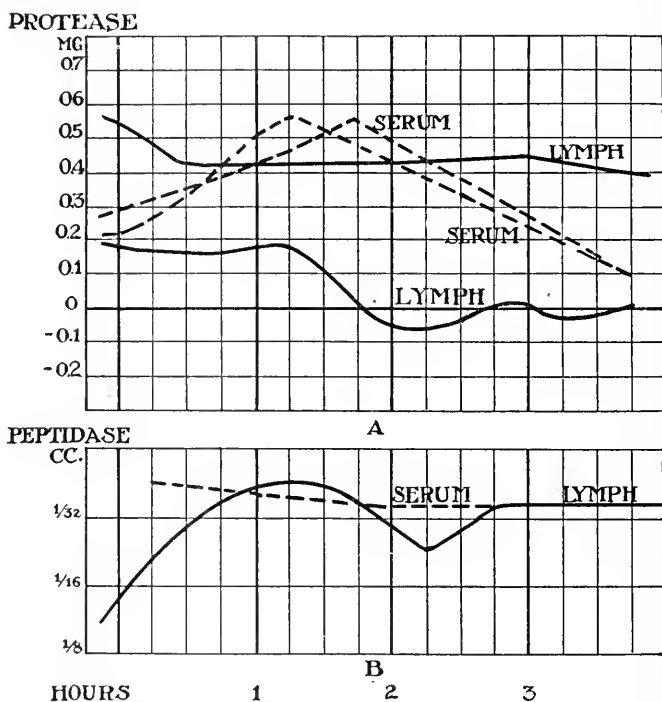
### EXPERIMENTAL.

Normal dogs of from 15 to 20 kilos were operated on as a rule in the morning, a lymph fistula was established, and recovery from the operation permitted. A liter of fresh milk was then fed. The lymph was completely collected in half hour periods; the blood was taken at various time intervals.

<sup>1</sup> Jobling, J. W., Petersen, W., and Eggstein, A. A., *J. Exp. Med.*, 1915, xxii, 129.

*Protease* (Text-Fig. 1).—The serum protease was found to increase uniformly until a maximum was reached between the 1st and 2nd hours after feeding, following which a progressive decrease occurred until in some animals no protease action could be determined in the serum.

The lymph protease decreased progressively following the feeding, in some experiments quite rapidly, so that the negative phase of the

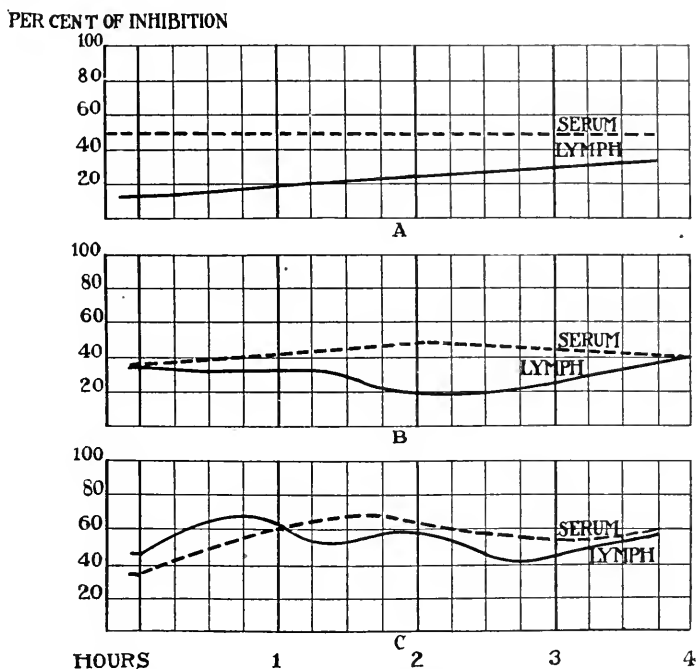


TEXT-FIG. 1. Effect of feeding on serum and lymph protease and ereptase (peptidase).

ferment action (proteosynthesis?) appeared within 2 to 3 hours after the feeding.

*Peptidase*.—The peptidase of the lymph is increased rapidly as a rule after the feeding, reaching a maximum at about the time that the maximum flow of lymph is observed. The peptidase of the serum, on the contrary, gave no evidence of an increase after the feeding (Text-fig. 1).

*Antiferment.*—In the previous experiments wide fluctuations were noted in the antiferment titer, which are readily understood when the fact that the antiferment titers of the serum and lymph are not necessarily equal is taken into consideration, so that the volume of the lymph flow entering the circulation appreciably modifies the antiferment titer of the serum.

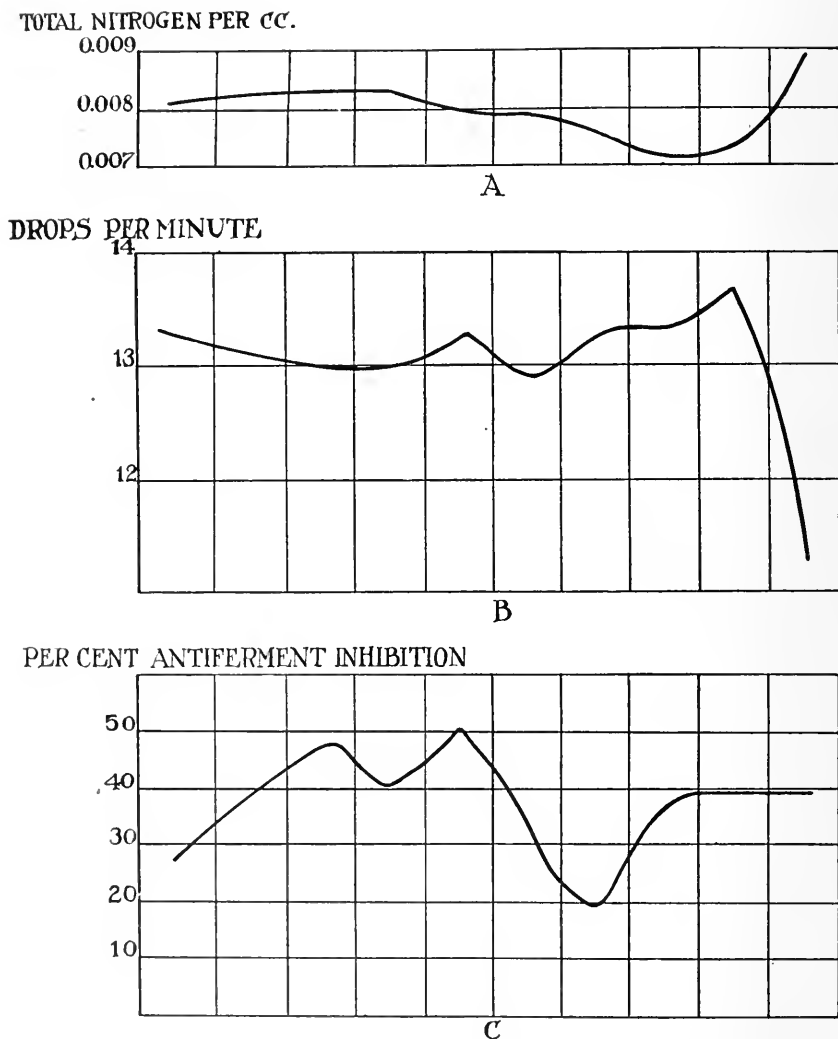


TEXT-FIG. 2. Effect of feeding on the antiferment titer of the lymph and serum.

The milk feedings used in these experiments caused a gradual increase in the antiferment titer of the lymph, but did not influence the titer of the blood serum (Text-fig. 2, A).

In the next experiment the milk fats were removed and 75 cc. of olive oil added to the milk in their place. It will be observed that in this case the antiferment titer of the lymph actually decreased, the serum showing a very slight increase (Text-fig. 2, B).

In Text-fig. 2, C is illustrated the effect of feeding 5 gm. of sodium



TEXT-FIG. 3. Relation of the antiferment to the lymph concentration and stalagmometric determination.

oleate, preceded by neutralization of the gastric acidity. In this case the anti-ferment both of the lymph and of the serum increased considerably.

*Relation of the Anti-ferment to the Lymph Concentration and Stalagmometric Determinations.*—Coincident with these experiments we have studied the relation of the anti-ferment to the concentration of the lymph as determined by the total amount of proteins contained, and also to the rate of flow through the stalagmometer. As will be observed in Text-fig. 3, the stalagmometric rate is influenced markedly by the concentration of the lymph. The anti-ferment on the other hand bears no relation to these two curves and its titer is obviously independent of the concentration of the proteins.

#### DISCUSSION AND SUMMARY.

In these experiments in which the lymph and serum ferments and anti-ferment have been studied separately, the changes that are found to occur are uniform and consistent. Possibly as a result of increased blood flow through the ferment-producing organs a moderate amount of protease is directly absorbed into the blood stream, but when intestinal digestion is actively under way this rapidly diminishes in extent. If any protease is absorbed during digestion from the gastrointestinal tract it is probably removed when it reaches the liver. The ereptase, or peptidase, is evidently absorbed directly from the intestinal tract and enters the circulation through the lymphatic channels.

The influence of the diet on the anti-ferment of the lymph is striking and accounts for the fluctuations observed in previous experiments. Following the milk meal the increase occurs gradually in the lymph in an amount that, when diluted in the blood stream, would be only nominal. When the fats of the milk were replaced by olive oil in large amounts it is surprising to find a decrease in the anti-ferment instead of an increase in titer, as might be expected in view of the nature of the anti-ferment. This result, however, is probably due to the fact that the anti-ferment lipoids of the serum and lymph may exist in both water and fat dispersion phases, but are active as anti-ferments only when in the former. If the amount of the fats of the

serum is increased, as it is after the olive oil feeding, more of the antiferment will enter the fat phase and will as a result be rendered inactive. When the feeding included the sodium oleate, the antiferment was in consequence increased in both the serum and the lymph, some of the soap being apparently absorbed directly into the blood stream. It is possible that the titer of the antiferment may be altered, therefore, by means of selective feeding.

## A COMPARATIVE STUDY OF LYMPH AND SERUM FERMENTS DURING PROTEIN SHOCK REACTIONS.

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(Received for publication, June 25, 1917.)

During the course of investigations concerning the mechanism of recovery following the so called "protein shock therapy" we have been interested in determining the comparative changes that occur in the lymph and blood serum and the possible manner in which these changes are brought about.

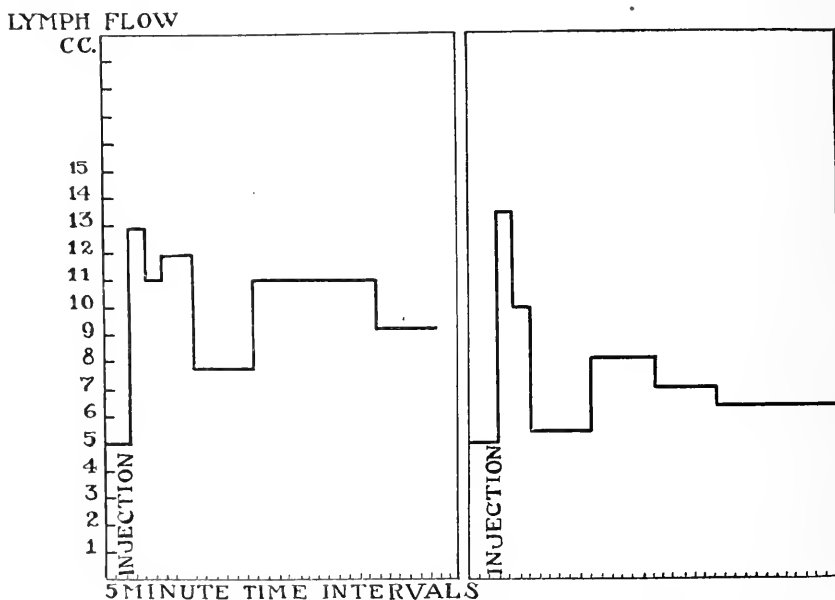
It is well known that certain substances, which Heidenhain (1) classified as lymphagogues of the first class, among them peptone, egg albumin, tissue extracts, etc., cause a marked increase in the lymph flow, supposed to be derived largely from the liver. This increased flow may continue a considerable time following such injection. Teague and McWilliams (2) have recently advanced the interesting explanation that this phenomenon is responsible for the therapeutic effect of the protein shock in that the antibodies of the blood are forced into the lymph spaces and there destroy the invading organisms. There is no doubt that the bacterial injections used in these experiments (*Bacillus coli*) do bring about a great augmentation of the lymph flow, as will be observed from the charts, and the explanation of Teague and McWilliams may well account in a large measure for the therapeutic effects.

### EXPERIMENTAL.

The technique used has been identical with that described in the previous papers. Dogs of from 20 to 40 kilos were anesthetized, a thoracic duct fistula was established, and after recovery from the operation killed colon bacilli were injected intravenously, the amount used for the shock varying from two to three slants of a 24 hour

agar culture. When necessary small doses of morphine were given, although we have endeavored to avoid such measures. If the animals are injected too soon after the operation and before complete recovery has been made from the anesthetic, considerable resistance to the shock may be manifest and the temperature reaction delayed for several hours.

*Lymph Volume.*—The increase in the rate of flow of the lymph follows immediately upon the injection (Text-fig. 1) and in severe



TEXT-FIG. 1. Volume of lymph flow following *Bacillus coli* vaccine injection.

intoxications two periods of maximum flow seem to occur, the first immediately after the injection and persisting for from 20 to 30 minutes, the second after approximately 1 hour, the latter increase being continued over a longer period of time. When the intoxication is not so great the two phase curve does not occur, the increase being less in extent but persisting for a longer period of time.

*Concentration of the Lymph and Serum and the Relation to the Stalagmometric Determination.*—The concentration of the lymph following the injection is considerably increased, as determined by the



Kjeldahl method for total protein nitrogen, although the concentration of the non-protein nitrogen may decrease, as shown in Table I.

TABLE I.

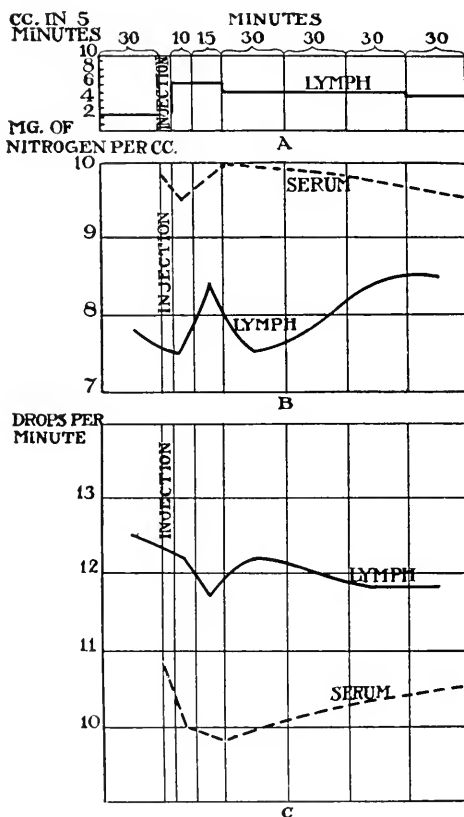
Time of sample.	Blood.		Lymph.	
	Total nitrogen per cc.	Non-protein nitrogen per cc.	Total nitrogen per cc.	Non-protein nitrogen per cc.
<i>a.m.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
11.30-12.00 (before injection) 12 n. 2 slants of colon bacilli injected.	9.8	0.45	7.8	0.5
<i>p.m.</i>				
12.05-12.15	9.5	0.50	7.5	0.45
12.15-12.30	9.7	0.45	8.4	0.45
12.30- 1.00			7.5	0.45
1.00- 1.30	9.8	0.50	7.8	0.47
1.30- 2.00			8.4	0.35
2.00- 2.30	9.5	0.50	8.5	0.35

The effect of the concentration of the lymph is observed directly in the rate of the stalagmometric flow, which is decreased when the concentration becomes greater. This does not always hold true for the blood serum, however, as shown in Text-fig. 2. A considerable diminution of the number of drops per minute was observed, without a corresponding increase in the concentration of the serum. Such a change must be due to an alteration in the dispersion of the serum colloids.

*Antiferment.*—In a preceding paper (3) it was shown that considerable fluctuations are to be observed in the antiferment titer of the lymph following feeding. The changes noted following protein shock make it probable that the antiferment is supplied to the blood stream wholly by way of the lymphatics, and not directly from the cells to the blood. In Text-fig. 3, A and B, are illustrated two curves of the antiferment index (0.05 cc. of serum and lymph) following intravenous bacterial injection.

In Text-fig. 3, A will be observed the prompt and marked rise in the antiferment of the lymph stream while the titer of the serum remains unaltered. Text-fig. 3, B shows a more gradual increase in the antiferment of the lymph with a coincident fall in the titer of

the blood serum. The increase in the antiferment observed in the lymph persisted in our experiments for the duration of the time under observation, usually about 5 hours. The increase probably would continue if the animal were permitted to live for a longer period

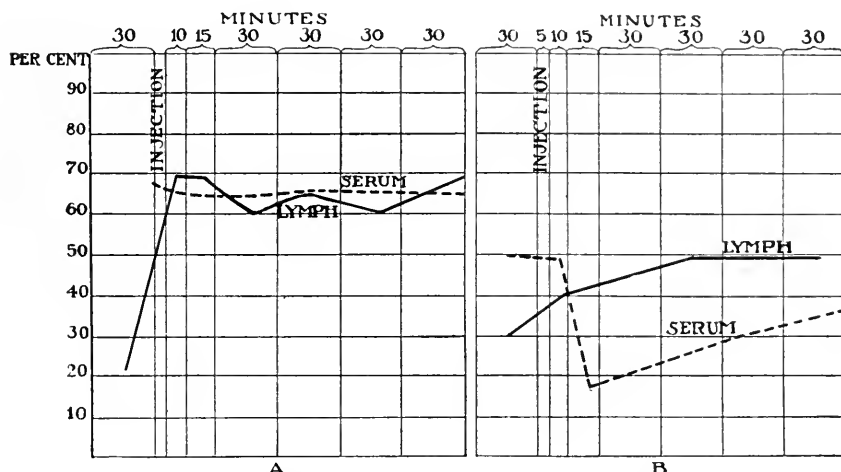


TEXT-FIG. 2. Relation of serum and lymph concentration and stalagmometric determinations. A, volume of lymph; B, concentration expressed as mg. of nitrogen per cc.; C, stalagmometric determination.

of time. No relation to the stalagmometric determination or to the protein concentration of the fluids was observed.

*Protease.*—The effect of the bacterial shock on the protease content of the lymph and serum is marked in extent. Three types of reaction may be distinguished: (a) the fluctuations in titer may

occur simultaneously, (b) those of the serum may precede those of the lymph, and finally, (c) there may be no relation of the one to the other, as illustrated in Text-fig. 4, C, in which the protease of the lymph increased and that of the serum diminished. Until more information concerning the origin and the distribution of these ferments in the vascular channels is available, it seems useless to endeavor to interpret these findings.

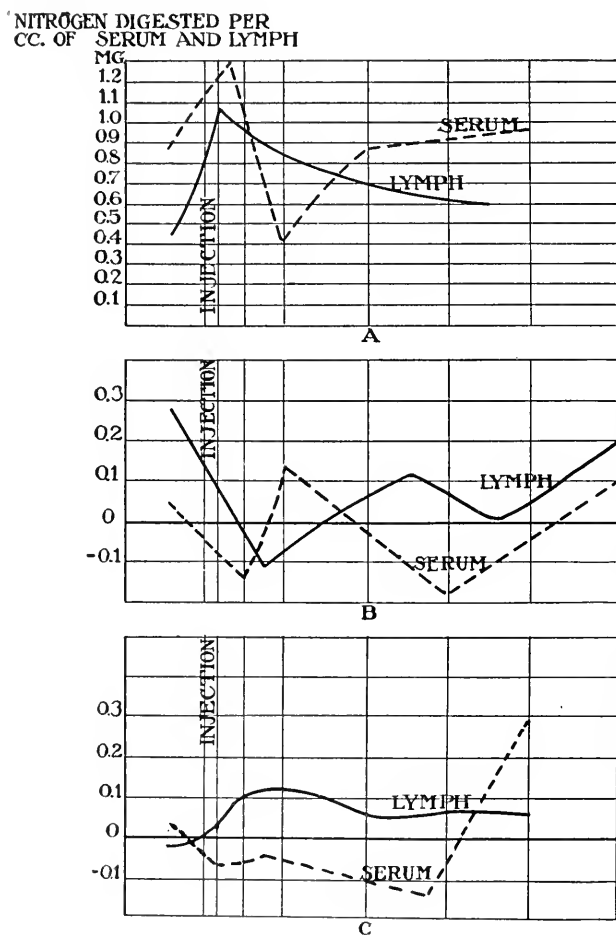


TEXT-FIG. 3. Antiferment of the lymph and serum following vaccine injections. A and B represent two different types of reaction.

*Peptidase.*—The fluctuations of the peptidase, or ereptase titer, do not parallel those of the protease; indeed the curves may be quite dissimilar. As a rule the increase makes its appearance later than that of the protease and is less extensive. When alterations in titer do occur they appear almost simultaneously in both the lymph and serum, although in a few experiments the ferment was first to be observed in the serum. It is at any rate apparent that the entrance into the blood stream can be direct and does not need to take place *via* the lymph channels, although under normal conditions, *i.e.*, feeding, this seems to be one portal of entry.

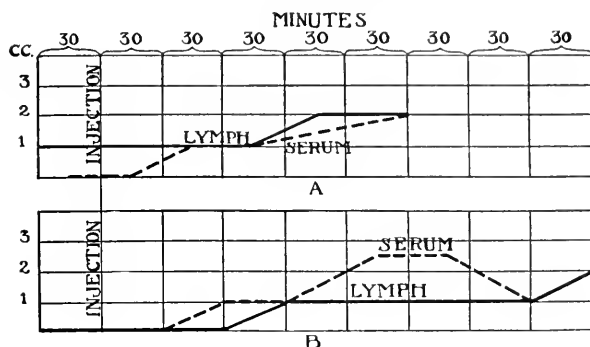
*Lipase.*—While the increase in this ferment occurs in both lymph and blood following shock, it seems to make its appearance first in the serum (Text-fig. 5). In Text-fig. 5, A it will be observed that

while the lymph titer was greater than that of the blood at first, the increase was first apparent in the serum, although the final titer



TEXT-FIG. 4. Protease titer following vaccine injections. A, B, and C represent three different types of reaction.

was equal in both lymph and serum. In Text-fig. 5, B is illustrated an experiment in which the increase occurred primarily and to the greatest extent in the serum.



TEXT-FIG. 5. Lipase titer following vaccine injections expressed in cc. of  $\frac{N}{50}$  sodium hydroxide. A and B represent two different types of lipase reaction.

*Diastase.*—The diastatic activity of both the serum and lymph remained practically equal and without change following the bacterial injections in all the experiments.

#### DISCUSSION AND SUMMARY.

The relation of two phenomena involved in the mechanism of recovery following protein shock therapy is shown in these experiments to be due to changes that concern the lymph rather than the serum. The first of these, the increase in the rate of flow of the lymph, has been suggested by Teague and McWilliams as a possible factor in recovery from infection when due to bacteria proliferating in the lymph spaces and inaccessible to the antibodies of the serum (typhoid). By means of the protein shock, antibody-rich fluids (serum) are forced into the lymph channels. That the antibodies of the serum are augmented following the shock has been demonstrated by Culver (4). With this possibility in mind it is to be expected that bacterial infection not confined to the lymph spaces will not be influenced by shock therapy to the same extent. How far this holds true we are unable to state, although von Decastello (5) has called attention to the fact that while he was able to cause rapid lysis or a crisis in typhoid patients following the shock reaction, the injection of a similar amount of vaccine in typhus fever was without effect. The second factor, the great increase in the anti ferment, is clearly due to the amount of the anti ferment entering the general

circulation through the lymph stream. This accounts for the marked fluctuations observed in the titer of the serum antiferment in patients following protein shock. If, for instance, the original titer of the lymph is less than that of the blood, the first flushing of the lymphatic current into the blood channel will tend to lower the titer of the serum, but with the increased amount in the lymph after the shock the titer of the serum will also increase.

Bacteria proliferate best where the antiferment is absent, as Wright (6) has noted in his studies on war wounds, a fact that is also commonly applied in bacteriological technique when we employ ascitic fluids, containing relatively little antiferment, preferably to serum, in culture media.

The increased lymph flow that follows the shock reaction would have value then, not only in forcing specific antibodies into the lymph channels, but in increasing the antiferment there as well, which would aid in checking the growth of bacteria.

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## STUDIES IN GLOMERULONEPHRITIS.

### III. AN ATTEMPT TO PRODUCE GLOMERULONEPHRITIS BY REPEATED INJECTIONS OF BACTERIA.

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PLATE 54.

(Received for publication, June 8, 1917.)

The present paper records an effort to reproduce glomerular nephritis in animals by inoculations of bacteria, especially by repeated inoculations such as might be expected to evoke phenomena of immunity, allergy, or sensitization. The indication for the attempt is found in the hypotheses put forth by several writers in explanation of diffuse glomerulonephritis, especially that occurring as a late sequel or complication of scarlet fever.

Volhard and Fahr state that it is characteristic of diffuse nephritis that it appears at a time when immune processes are active and speak of a "diffuse toxic action." Ophüls has suggested that the glomerular endothelium acquires strong bacteriolytic properties which cause an "explosive" reaction when the bacteria are again introduced into the tissue, lesions being caused by disruption products of the bacteria. The most elaborate development of the theory is that of von Pirquet and Escherich and Schick, who apply it also to the other late complications of scarlet fever. It may be summarized as follows: At the beginning of the disease, during the acute symptoms, the virus circulates in the body, sensitizing the cells in the same way as do all the foreign proteins. The immune substances (ergins) begin to appear at the end of the 2nd week. At this time a reinvasion of the body from various foci occurs, a specific reaction or combination between antigen and antibody takes place, and this, after the latent period common to allergic reactions in general, leads to active signs and symptoms of the scarlatinal sequelæ. The latter actually occur in the 3rd and 4th weeks, between the 19th and 22nd days. It is also part of the theory that the smaller the amount of protein acting in the preliminary sensitization, the sharper will be the later allergic reaction.

Von Pirquet and Escherich and Schick state that in the case of scarlet fever experimental proof of the allergic hypothesis must await the discovery of the

specific virus of the disease, but Löhlein asserts that streptococci are either demonstrable or probably associated in all cases of diffuse nephritis, and it is commonly accepted (Holt and Howland) that many of the complications of scarlet fever, including nephritis, are probably due to secondary infection with streptococci.

It therefore seemed logical to attack this problem, in a way somewhat like that in which arthritis was previously studied, by investigating the effect upon the kidney of repeated inoculations of streptococci. In view of the fact that streptococci cause the production of only small amounts of recognizable antibodies in the serum, it was conceived that a more decisive test of the part played by humoral bacteriolysis<sup>1</sup> at least, would be furnished if bacteria (*Bacillus coli communior*) capable of evoking large amounts of serum antibodies were used. As a still further test, the effect of repeated large doses of these bacteria in a highly immunized and therefore resistant animal was investigated. Finally, several strains of *Staphylococcus aureus* were used for repeated inoculation.

#### EXPERIMENTAL.

With the exception of *Bacillus coli communior*, all the bacteria used in the following experiments were isolated from the throats of patients in the 1st week of scarlet fever. The first inoculation was usually from the second or third generation. In general, the organism found in greatest abundance was used. As a rule this was the streptococcus which was found in a majority of instances to be of the non-hemolytic type (*Streptococcus salivarius*). In a few instances a predominant growth of *Staphylococcus aureus* was obtained and once the Klebs-Loeffler bacillus outgrew the other varieties.

Rabbits were used exclusively, usually young females weighing from 1,200 to 1,800 gm.

All tissues were fixed for routine examination in Orth's solution, and in formalin for frozen sections and for sections to be stained for bacteria

<sup>1</sup> Here and elsewhere in this paper the term bacteriolysis is used to imply the proteolysis that is assumed to occur when bacterial antigens are subjected to the action of complement and antibody. Jobling and Petersen have recently shown that the phenomenon may be rather a process of solution than of proteolytic cleavage.



by the Giemsa method. All sections, except the last named, were stained by Van Gieson's method.

Giemsa stains were used on nearly all the kidneys, which were carefully examined, without success, for bacteria.

The results of the experiments are given in Table I.

In general the glomerular changes were strikingly slight, though a majority showed one or more of the minor changes commonly found in glomerular nephritis, such as swelling of the endothelium, increase of the endothelial nuclei, and small amounts of granular exudate in the capsular space. If the data are examined closely it will be seen that the extent of such changes bears no appreciable relation to the number of injections or the character of the bacteria injected. Lesions are perhaps somewhat more frequent in those animals which died spontaneously than in those which were killed. In the one or two instances where a true albuminous exudate into the capsular space occurred a coincident spontaneous nephritis was observed. This complicates matters and renders generalizations hazardous. Leaving out these cases, we are at the most justified only in saying that bacterial inoculation produced a very mild grade of intraglomerular damage, but nothing comparable to the diffuse extraglomerular nephritis seen in man, with its exudate of fibrin, serum, and cells into the capsular space.

For bacteriolytic experiments, organisms of the typhoid-colon group are, as is well known, much better suited than streptococci, and it was for this reason that the experiments of Series D with *Bacillus coli communior*, which is highly virulent for rabbits, were carried out. In at least three, and probably all, of the animals of this series, a high degree of immunity, measured in terms of agglutinins was induced (Table II). After immunization it was possible to inject such massive doses of bacteria as in untreated animals would cause sudden death. It was assumed on the basis of the modern belief in the unity of the so called sensitizer antibodies (Zinsser) and our agglutination tests that the animals had acquired greatly heightened powers of bacteriolysis and, hence, that the massive inoculations employed were followed by an inundation of the body with split bacterial protein. Indeed, marked prostration followed the injections and was taken as direct evidence of proteotoxic action. The glomerular changes in this series were perhaps slightly

TABLE I.  
*Renal Changes after Repeated Intravenous Injections of Bacteria.*

Rabbit No.	Original weight of animal.	Culture used.	No. of injections.	Amounts of culture injected.	Day of experiment on which injections were given.	Interval between last injection and death.	Died or killed.	Combined weight of kidneys.	Renal changes.
Series A, <i>Streptococcus hemolyticus</i> .									
gm.	cc.	gm.	days	gm.					
1	1,400	No. 1	4	1.0, 1.0, 1.0, 1.0	1, 5, 6, 7	2 hrs.	D.	9.9	Moderate increase of endothelial cells, some with pyknotic nuclei. Granular material in capsular space.
2	1,500	" 1	4	2.0, 2.0, 2.0, 2.0	1, 5, 6, 7	10	"	9.6	Slight desquamation of epithelium of Bowman's capsule.
3	1,500	" 1	4	0.05, 0.05, 0.05, 0.05	1, 5, 6, 7	9	"	8.6	Occasional red blood cells in capsular space. Occasional dilated loop in tufts. Changes slight.
4	1,500	" 1	4	0.02, 0.02, 0.02, 0.02	1, 5, 6, 7	5	"	9.0	Slight increase of endothelial cells and in places stratification of parietal layer of Bowman's capsule.
5	1,750	" 1	3	1.0, 1.0, 1.0	1, 2, 3	14	"	12.1	Considerable increase of endothelial cells of tufts. Some granular exudate in capsular space. Some hyaline casts.
6	1,750	" 1	3	2.0, 2.0, 2.0	1, 2, 3	18	"	10.0	A few glomeruli show increased endothelial cells.
7	1,200	" 1	5	10M., * 10M., 10M., 100M., $\frac{1}{3}$ Bl.	1, 2, 3, 33, 44	12	K.	10.8	Endothelium of glomeruli slightly swollen in places. No other notable changes.
8	1,800	" 1	3	10M., 10M., 10M.	1, 2, 3	4	D.	15.2	No notable changes.

9	1,600	No. 1	5	10M., 10M., 10M., 100M., $\frac{1}{3}$ Bl.	1, 2, 3, 33, 44	12	K.	12.0	Moderate endothelial swelling. dilated empty loops.	Occasional
10	1,750	"	1	20M., 40M., 80M., 100M., $\frac{1}{3}$ Bl.	1, 2, 3, 26, 37	12	"	16.8	Moderate endothelial swelling. empty loops, not dilated.	Occasional
11	1,700	"	1	20M., 40M., 80M., 100M., $\frac{1}{3}$ Bl.	1, 2, 3, 26, 37	12	"	14.5	About same as No. 10.	
12	600	"	1	$\frac{1}{8}$ Sl., $\frac{1}{8}$ Sl., $\frac{1}{4}$ Sl., $\frac{1}{4}$ Sl., $\frac{1}{2}$ Sl., $\frac{1}{2}$ Sl.	1, 2, 3, 4, 5, 6	8	D.	4.7	Considerable granular exudate in capsular space.	
13	750	"	1	$\frac{1}{8}$ Sl., $\frac{1}{8}$ Sl., $\frac{1}{4}$ Sl., $\frac{1}{4}$ Sl., $\frac{1}{2}$ Bl.	1, 2, 3, 4, 5	12	K.	7.8	Considerable endothelial proliferation and empty loops.	
14	750	"	1	$\frac{1}{8}$ Sl., $\frac{1}{8}$ Sl., $\frac{1}{4}$ Sl., $\frac{1}{4}$ Sl., $\frac{1}{2}$ Sl., $\frac{1}{2}$ Sl., $\frac{1}{3}$ Bl.	1, 2, 3, 4, 5, 6, 7	12	"	10.3	Slight endothelial proliferation and some empty loops.	
15	—	"	S 1	6.0	1	$\frac{1}{2}$	D.	9.5	Leukocytes in glomeruli. Endothelial nu- clei stain deeply.	
16	1,300	"	S 5	10.0, 6.0	1, 10	2	"	13.2	Glomeruli enlarged. Abundant granular and reticular exudate in capsular spaces.	
17	2,775	"	Ch 1	5.0, 5.0, 5.0, 5.0, 5.0, 6.0	1, 6, 12, 19, 27, 33	11	K.	15.3	Endothelial nuclei stain deeply. Moderate swelling and nuclear increase of glomerular endothelium. Yellowish pig- ment granules in epithelium and lumen of distal convoluted tubules.	
18	3,425	"	S 16	5.0, 5.0, 5.0, 5.0, 5.0, 6.0	1, 6, 13, 19, 27, 33	11	"	24.0	Marked swelling and nuclear increase of glomerular endothelium. Leukocytes in tufts increased. Many hyaline casts.	
19	2,100	"	S 17	5.0, 5.0, 5.0, 5.0, 5.0, 6.0	1, 6, 12, 19, 27, 33	11	"	15.4	Moderate swelling and nuclear increase of glomerular endothelium.	
20	1,425	"	N 1	6.0, 6.0, 6.0, 5.0, 5.0, 5.0, 6.0	1, 38, 44, 50, 57, 65, 71	11	"	—	Practically normal.	

\* Killed by heat (60°C., 1 hour).

† M. indicates million bacteria; Bl., Blake bottle, agar culture; Sl., agar slant culture. All doses are given in cc. of a 24 hour broth culture, unless otherwise specified.

TABLE I—Continued.

Rabbit No.	Original weight of animal	Culture used.	No. of injections	Amounts of culture injected.	Day of experiment on which injections were given.	Interval between last injection and death.	Died or killed.	Combined weight of kidneys.	Renal changes.
Series B, <i>Streptococcus viridans</i> .									
	gm.	No.		cc.		days		gm.	
21	1,450	No. S 6	3	14.0, 5.0, 5.0	1, 11, 38	5	D.	12.2	Glomeruli swollen and bloodless. Capsular space obliterated. Endothelial nuclei increased in places. Practically normal.
22	1,450	" S 7	1	6.0	1, 28	12	"	—	No notable changes.
23	1,540	" S 9	2	6.0, 6.0	1, 6, 12, 19, 27, 33	4	"	10.5	"
24	2,900	" S 6	6	6.0, 5.0, 6.0, 5.0, 5.0, 6.0		11	K.	16.3	"
Series C, <i>Staphylococcus aureus</i> .									
		No.							
25	2,050	No. S 8	1	6.0	1	1	D.	19.5	Glomeruli large. Endothelium markedly swollen. Occasional leukocytes in capsular space. Many hyaline casts.
26	2,100	" S 10	1	6.0	1	1	"	17.2	No notable changes except spontaneous nephritis.
27	2,700	" S 10	5	2.0, 5.0, 5.0, 3.0, 3.0	1, 7, 12, 19, 34	10	K.	15.5	Glomeruli greatly congested, but endothelium not swollen. Nuclei somewhat increased. Occasional red blood cells in capsular space.
28	2,700	" S 12	6	2.0, 5.0, 5.0, 3.0, 3.0, 3.0	1, 7, 12, 19, 27, 34	10	"	14.2	No notable changes.
29	2,725	" S 10	6	2.0, 5.0, 3.0, 3.0, 3.0, 3.0	1, 7, 12, 19, 27, 34	10	"	16.8	Moderate increase of endothelial nuclei. No other notable changes.

Series D, *B. coli communior*.

30	1,700	<i>B. c. c.</i>	5	10M., 10M., 10M., 100M., 1.0	1, 2, 3, 33, 44	12	K.	15.4	Dilated empty loops and swollen epithelium in many tufts. Occasional pyknotic nuclei. Considerable granular exudate in capsular space.
31	1,500	"	5	10M., 10M., 10M., 100M., 1.0	1, 2, 3, 33, 44	12	"	10.7	Moderate swelling and multiplication of endothelium. Occasional dilated empty loops.
32	1,600	"	4	20M., 40M., 80M., 100M.	1, 2, 3, 26	10	D.	13.1	Marked increase of endothelial nuclei. No other marked change.
33	1,600	"	5	20M., 40M., 80M., 100M., 1.0	1, 2, 3, 26, 37	12	K.	12.2	Many empty loops but no marked endothelial swelling.
34	1,250	"	1	1 $\frac{1}{10}$ Bl.	1	2	D.	—	General capillary and venous engorgement. Occasional red blood cells in capsular space. A few hyaline casts.
35	1,600	"	10	1 $\frac{1}{10}$ Bl., 1 $\frac{1}{10}$ Bl., 1 $\frac{1}{10}$ Bl., $\frac{1}{3}$ Sl., $\frac{2}{3}$ Sl., 1 Sl., 2 Sl., $\frac{1}{3}$ Bl., $\frac{1}{3}$ Bl.	1, 6, 12, 20, 21, 22, 23, 24, 25, 27	3	"	13.0	Very marked spontaneous nephritis. In fibrotic areas, many of the capsular spaces of the glomeruli contain yellow-staining albuminous exudate, and several others are greatly dilated and contain a non-staining homogeneous exudate. Most of the glomeruli are enlarged, bloodless, with swollen epithelium. Many eosinophils in glomerular and intertubular capillaries. No bacteria found.
36	1,060	"	7	1 $\frac{1}{10}$ Bl., 1 $\frac{1}{10}$ Bl., 1 $\frac{1}{10}$ Bl., $\frac{1}{3}$ Sl., $\frac{2}{3}$ Sl., $\frac{1}{3}$ Sl.	1, 6, 12, 20, 21, 22, 23	1	"	12.4	Glomeruli enlarged with many dilated empty loops. Endothelium swollen. Some pyknotic nuclei. No exudate in capsular spaces.
37	1,460	"	9	1 $\frac{1}{10}$ Bl., 1 $\frac{1}{10}$ Bl., 1 $\frac{1}{10}$ Bl., $\frac{1}{3}$ Sl., $\frac{2}{3}$ Sl., $\frac{1}{3}$ Sl., 1 Sl., 2 Sl., $\frac{1}{3}$ Sl.	1, 6, 12, 20, 21, 22, 23, 24, 25	1	"	14.1	Glomeruli moderately enlarged. Slight granular exudate occasionally found in capsular spaces but no marked or extensive glomerular changes.

more marked than in the series in which streptococci and staphylococci were used, but again they failed to correspond in intensity with the number or the amounts of the injections. Thus, Rabbit 37, which received six large injections, suffered but a small amount of glomerular injury. On the whole, it is difficult in our experiments to find support for the bacteriolytic theory of glomerulonephritis.

The fate of bacteria in the kidney is a matter of some interest, but could not be fully investigated in the present work. However, a few observations bearing on this point were made in connection with experiments, previously reported, when successive injections of diphtheria toxin and *Bacillus coli communior* were given. Large amounts of bacteria injected intravenously led to rapid death of the animal. The bacilli were found in dense clumps (Fig. 1), mostly in the glomeruli and

TABLE II.

*Agglutination Tested for Rabbits 35, 36, and 37 on the 17th Day after the First Injection.*

Rabbit No.	Dilution.										
	1:25	1:50	1:100	1:250	1:500	1:1,000	1:2,500	1:5,000	1:10,000	1:25,000	1:50,000
35	=	+	+	++	+++	+++	++	+	+	=	0
36	+	+	+++	++	+++	+++	+	++	=	++	+++
37	+	+	+	++	++	+++	++	++	+	++	+

occasionally in the intertubular capillaries of the cortex and medulla. In these animals few leukocytes and little evidence of inflammatory reaction were found in the tufts. In animals dying 12 to 48 hours after inoculation the bacteria had usually disappeared and leukocytes, generally in large numbers, were found in the loops. In most of these animals there was a rather marked inflammatory reaction in the glomeruli. It was at first thought that a local bacteriolysis, by leukocytes, with the formation locally of poisonous bacterial disintegration products might be the cause of the inflammatory reaction, but against this was the fact that even with enormous doses (sediment of 30 cc. of broth culture) only a few glomeruli contained demonstrable organisms, whereas the inflammatory reaction involved a majority of the tufts. It was therefore assumed that the toxic substances with their diffuse action,

were circulating in the blood. It does, however, seem fairly probable that the bacteria were occluded and removed by leukocytes, and in one instance this was directly observed (Fig. 2).

The theories which we are examining are closely dependent upon an assumed bacteriolysis of streptococci and it is necessary to examine closely the question whether lysis of the streptococcus can experimentally be shown to occur. To such physical and chemical procedures as freezing and thawing, and extraction with distilled water and weak alkali the streptococcus has, in my hands, proved to be extraordinarily resistant and experiments to be described below indicate that it is correspondingly resistant also to lysis by the Pfeiffer procedure, and by immune serum and complement. The contrast in these respects with the typhoid-colon group is, in fact, so great as to suggest a division of bacteria into what might be called lysostable and lysolabile groups.

In a previous paper (Faber) on experimental arthritis in rabbits it may be recalled that injection of streptococci into the knee-joints of rabbits was followed by a condition designated as sensitization, which was revealed by the occurrence of a local reaction when homologous organisms were later injected intravenously. The term sensitization was purposely chosen to indicate altered properties of the cells of the capsular synovium, without offering a more explicit interpretation of the exact process, whether it was bacteriolysis with the local formation of toxic bacterial disruption products, or an increase in the normal defensive leukocyte-attracting properties of the cells, or a process related in some way to a disturbed cellular ferment balance consequent to local specific antibody-antigen combinations. In point of fact, certain unpublished experiments were carried out at that time in an attempt to elucidate the problem in one of these directions, and may be cited here.

#### *Pfeiffer's Phenomenon for Joints.*

*Rabbit 40.*—Left knee injected with mixture of 0.1 cc. of immune serum + 1 loop of *Streptococcus viridans* + 0.9 cc. of 0.85 per cent salt solution.

Right knee injected with mixture of 1 cc. of sterile bouillon + 1 loop of *Streptococcus viridans*.

Both knees aspirated after 1 hour. Cultures: left knee, heavy growth of streptococcus; right knee, no growth.

*Rabbit 41.*—Left knee injected with mixture of 0.1 cc. of normal rabbit serum + 0.9 cc. of salt solution + 1 loop of *Streptococcus viridans*.

Right knee injected with mixture of 1 cc. of bouillon + 1 loop of *Streptococcus viridans*.

Both knees aspirated after 1 hour. Both cultures sterile.

The immune serum was obtained from a rabbit which had received two injections of living streptococcus. Complement fixation was positive in the serum. This rabbit developed an acute arthritis immediately after receiving a third injection (shortly after the time that blood for the above serum was drawn).

In connection with the above experiments, the following cultural tests were made.

(a) 1 loop of *Streptococcus viridans* + 1 cc. of immune serum. Incubated 1 hour and plated. Result scanty growth.

(b) 1 loop of *Streptococcus viridans* + 1 cc. of normal serum. Incubated 1 hour and plated. Result heavy growth.

(c) 1 loop of *Streptococcus viridans* plated. Result heavy growth.

These experiments, though too few to be decisive, suggest the following conclusions. (1) Immune streptococcus serum not only fails to evoke the Pfeiffer phenomenon, but may even favor the bacteria at the expense of the tissues. (2) The normal synovial membrane has considerable power to combat infection. (3) Immune serum apparently has the power of partially inhibiting growth *in vitro*.

The third point might be used as an indication of bacteriolysis and was therefore tested more completely in another series of experiments which are given in Table III.

The following points may be emphasized. (1) The lowest counts were obtained in those plates in which the dilution of the serum had been respectively 1:30 and 1:50. The maximum agglutination was obtained with a dilution of 1:40. (2) The lowering of the bacterial count was independent of the amount of complement added. (3) Lowering of the bacterial count was obtained without complement and in two instances was greater than in any of the tubes to which complement had been added.

These experiments strongly suggest that we are dealing not with bacteriolysis proper but with an agglutination phenomenon. It is



obvious that an agglutinated clump of bacteria will give but a single colony and that agglutination in this way will cause an apparent reduction of the bacterial count when the pour-plate method is used.

TABLE III.

*Tests for Bacteriolysis with Streptococcus viridans, Complement, and Immune Serum.*

Complement.	Row.	Cc. of immune serum.						
		0.1	0.08	0.05	0.03	0.01	0.007	0.00
		Dilution.						
		1:15	1:20	1:30	1:50	1:150	1:200	—
		Tube.						
cc.		1	2	3	4	5	6	7
0.5	A	180	150	125	180	225	180	300
0.3	B	170	250	105	110	200	180	
0.1	C	160	160	75	40	140	160	
0.07	D	125	150	90	150	150	175	
0.04	E	90	150	110	19	100	110	
0.00	F	160						375

The figures represent thousand colonies per plate.

To each tube was added 0.5 cc. of a suspension of streptococci containing 750,000 bacteria per cc. All the tubes before incubation were made up to equal volume with 0.85 per cent sodium chloride solution.

#### DISCUSSION.

It may be assumed that when bacteria are introduced into a tissue the mechanism of defense is one of physical removal, depending on the leukotactic activity of the tissue or bacteria in question (Fig. 2). It may also be assumed that this activity is heightened by previous exposure of the tissue to the infecting agent and that the reaction will usually be accompanied by such other reactive phenomena as vasodilation and exudation of fluid. Bacteriolysis of streptococci *in situ* and the local production of toxic disintegration products is at the least an improbable explanation of the phenomenon.

The conclusions to be derived from the experiments on joints may perhaps fairly be applied to the kidney, save that in the latter we have obtained much less evidence of reactivity. It is evident that the

arrangement of the glomerular capillaries is such that small bacterial emboli can be expeditiously removed by phagocytes and that there is in the tuft no closed space in which inflammatory exudate can collect. It may, indeed, be doubted whether these emboli are permitted to remain long enough in contact with the glomerular endothelium to set up a state of sensitization. In none of the rabbits examined were bacterial emboli found more than 12 hours after injection and then in only one instance when the animal had been profoundly intoxicated with diphtheria toxin.

The capsular space, on the other hand, does supply conditions of relatively poor drainage which are increased when the excretion of water from the tuft is interfered with. Apparently, it is when the lining epithelium of Bowman's capsule has been damaged and permits toxic substances to pass through that leukocytes, fibrin, and the other materials of inflammatory exudate are attracted to the capsular space and so produce the picture of extraglomerular nephritis. This picture, it will be recalled, was produced when an injection of bacteria followed injury by diphtheria toxin. The present series of experiments with their consistently negative results constitutes strong evidence that bacteria alone are unable to produce extraglomerular nephritis, even by cumulative action or by immune bacteriolysis, since on the one hand a bacterial species known to be subject to immune bacteriolysis failed to produce the lesions under favorable conditions, and since on the other hand the organism commonly found in this disease has been shown to be extraordinarily resistant to bacteriolysis.

The evidence of the present experiments and the arguments presented against the bacteriolytic theory would also appear to apply equally well to the assumption of a local allergic reaction in the glomerular endothelium.

Embolism, even in an immune host where agglutination *in vivo* may be assumed to occur, is too sporadic in the kidney to explain lesions as widespread and evenly distributed as occur in the human disease, and it seems more than ever necessary to assume the action of a soluble poison in the blood as the immediate pathogenetic factor. For the present there is no direct proof of the nature or of the origin of this poison.

The local concentration of poisons in the tuft, whatever may be their source or nature, may, however, still be held to be the immediate cause for the occurrence of lesions at this point.

#### SUMMARY.

Repeated injections into the blood stream of streptococci and staphylococci derived from cases of scarlet fever, and of *Bacillus coli communior* failed to produce typical glomerulonephritis even when immune antibodies could be demonstrated in the serum in high dilutions. Bacteriolysis of streptococci was not found by the usual tests *in vitro* or by the Pfeiffer procedure. It is therefore concluded that the weight of evidence is against the theory that glomerulonephritis is due to immune bacteriolysis of streptococci. The experiments also failed to give any support to the hypothesis of allergy or of sensitization as a factor in the production of the disease. Evidence is presented to show that bacterial emboli are rapidly removed from the glomerular capillaries by leukocytes, and that this embolism, even after injections of enormous quantities of bacteria, affects but a small proportion of the glomeruli. It is again suggested that a circulating poison in the soluble state is responsible directly for the disease in question.

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## EXPLANATION OF PLATE 54.

FIG. 1. Rabbit 38. Intravenous injection of the sediment of 30 cc. of a broth culture of *B. coli communior*. Died 1 hour later. Large bacterial embolus in glomerulus and its afferent vessel. Giemsa stain. Leitz obj. 6.

FIG. 2. Rabbit 39. Intravenous injection of one agar slant of *B. coli communior*. Died 12 hours later. Bacterial embolus in glomerulus. Adjacent to this is a polymorphonuclear leukocyte containing a bacillus. Giemsa stain. Obj. oil immersion.

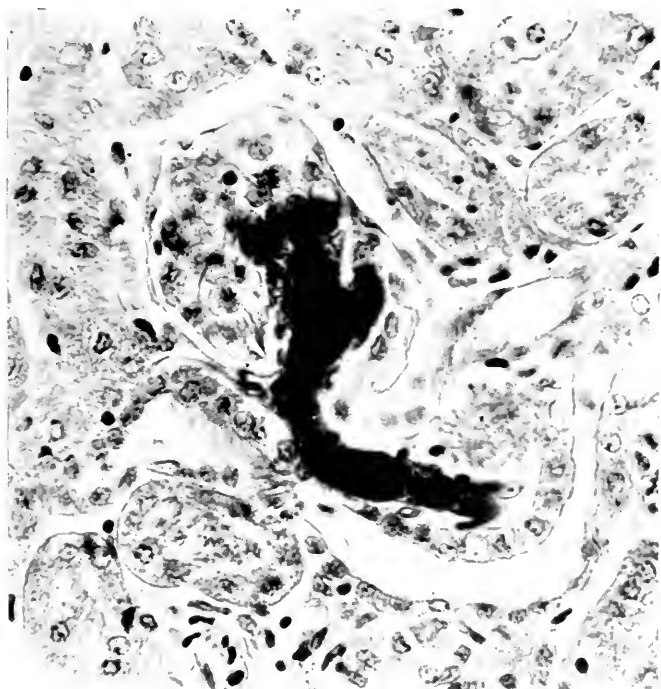


FIG. 1.

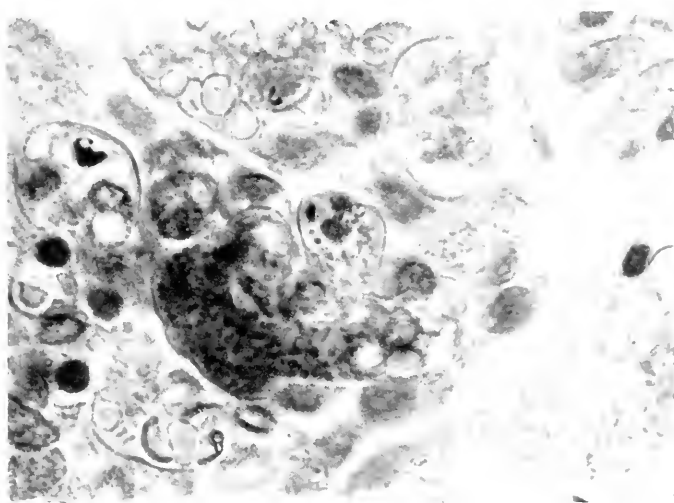


FIG. 2.

(Faber and Murray: Studies in glomerulonephritis. III.)



# THE INTERRELATION OF THE SURVIVING HEART AND PANCREAS OF THE DOG IN SUGAR METABOLISM.

## SECOND PAPER.

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The clue which led to the present investigation was the discovery that when a Locke's solution containing dextrose was perfused through the pancreas of a dog, there was a distinct diminution in the optical rotation of the perfusate but no change in its reducing properties. This result, which I had not anticipated, seemed of interest, for it would indicate that the perfused pancreas exerts an independent effect on dextrose, a fact which had not been evident from the reducing properties alone. The following experiments were carried out in an attempt to analyze the nature of this effect and to study further the general problem of the interrelation between the heart and pancreas in sugar metabolism with especial relation to their specific action, separately and combined, on dextrose and levulose. The results have been definite and have led to a tentative hypothesis which at present seems best suited to explain the facts that have been obtained.

In the previous investigation (1) it was found when a pancreas was perfused aseptically with Locke's solutions containing physiological concentrations of dextrose that, while there was no change in the reducing properties of the perfusate, yet the pancreas supplied something to the perfusate which subsequently brought about a utilization of sugar by the living heart to an extent that did not occur with the heart alone. This pancreatic substance, as far as could be determined, possessed the characteristics of an enzyme. It was inactivated by boiling; it acted in small amounts; it was unstable, rapidly becoming inactive on standing; it caused a great acceleration in the rate of a reaction which otherwise proceeded slowly, and the rate of reaction diminished as the reaction proceeded. The living heart in the presence of this pancreatic factor was responsible for two effects: first, a change of the sugar to a non-reducing form that yielded again a simple sugar on hydrolysis or by simply standing with a preservative at 37°C. for 24 hours; second, a disappearance of sugar which was probably due to its destruction by hydrolysis or oxidation.

*Methods.*

Briefly outlined, the methods of approaching the problems in the present work were by perfusing the living heart and pancreas with oxygenated Locke's solutions containing dextrose and levulose. With these factors, the actual combinations which were used are given below:

- |   |                                  |
|---|----------------------------------|
| (a) Pancreas + dextrose.                    | (d) Heart + dextrose.            |
| (b) Pancreas + levulose.                    | (e) Heart + levulose.            |
| (c) Pancreas + dextrose + levulose.         | (f) Heart + dextrose + levulose. |
| (g) Pancreas + heart + dextrose.            |                                  |
| (h) Pancreas + heart + levulose.            |                                  |
| (i) Pancreas + heart + dextrose + levulose. |                                  |

With these tissue and sugar combinations, the original Locke's solutions were compared with the final perfusates, both before and after hydrolysis, as to their optical rotation, reducing power, and, in a number of experiments, the melting points of the osazones.

*Perfusions.*—The same method of isolated perfusion and Locke's solutions of the same composition were used as in the former experiments (1), and the same aseptic technique and standard of asepsis were rigidly maintained. No heart perfusion was considered in this series where the heart did not continue to beat actively over the period of perfusion. The bacterial counts given in the tables show that exclusion of bacteria was even more successful than formerly.

*Sugars.*—The sugars were dextrose (Kahlbaum, pure) and levulose (Kahlbaum, pure, from inulin). In a few of the early experiments, Schering's crystalline powdered levulose was used, but as it showed no difference in its action from the Kahlbaum levulose which was subsequently obtained, the experiments are included. The sugar in each experiment was dissolved and boiled for at least 10 minutes before adding it to the Locke's solution, to eliminate any phenomenon of mutarotation. With an occasional exception the concentrations of sugar in the original Locke's solutions were from 0.3 to 0.4 per cent.

*Sugar Determinations.*—The quantitative sugar determinations were made both by the colorimetric method of Lewis and Benedict (2) and with the polariscope. The preliminary procedure was as follows: 50 cc. each of the original Locke's solutions and of the perfusates were measured in volumetric flasks and each portion was



added to 1 gm. of trichloroacetic acid carefully weighed from the same stock bottle. This amount of reagent gave a complete precipitation of whatever protein was present in the perfusates, and on filtration yielded a clear fluid. To each filtrate sufficient concentrated hydrochloric acid was added to give the desired per cent of acidity. As far as could be ascertained, a hydrolytic effect was obtained with dextrose solutions when either 1 per cent or 0.5 per cent hydrochloric acid was used, while with levulose 0.5 per cent hydrochloric acid could not be exceeded without sugar destruction. From these acidified specimens samples were immediately taken for sugar determination. The samples taken for the polariscope were returned and the solutions placed in a boiling water bath for an hour with reflux condensers. At the end of this time they were cooled and specimens were again removed for determinations of optical rotation and reduction. In each experiment the amount of reducing sugar was based on the polariscopic determination of the original Locke's solution. By this procedure the reducing sugar and optical rotation were measured before and after hydrolysis in the original Locke's solution and in the perfusates under constant conditions of volume and acidity.

The possibility was suggested at first that in precipitating the protein a portion of the sugar might be carried down. In control experiments, however, as shown in the tables, this did not occur. In these controls even with the precipitation of a relatively large amount of protein the filtrate did not appreciably vary from the original Locke's solution, either in rotation or in reduction. The addition of trichloroacetic acid and hydrochloric acid increased the volume of fluid slightly and so diminished the sugar per cent. However, since this increase in volume is constant for all samples, the significance of comparative determinations remained unchanged.

The polariscopic determinations were made with a Schmidt and Haensch polariscope which gives a direct reading to the hundredth of a degree. In each experiment all readings were made in a 4 dm. tube under constant temperature conditions, and the final reading was taken as the average of five successive determinations not varying over  $0.02^\circ$ . This gave results which could be compared with a fair degree of accuracy to the third decimal place. The specific

rotation of dextrose was taken as  $+52.5^\circ$ , and of levulose as  $-91^\circ$ . The controls gave a final check on the accuracy of the results.

TABLE I.  
*Hydrogen Ion Concentrations of Perfusates.*

Experiment No.	Tissues perfused.	Duration of perfusion.	pH Locke's solution.		pH perfusate.		Sugar used
			Carbon dioxide present.	Carbon dioxide removed.	Carbon dioxide present.	Carbon dioxide removed.	
		<i>hrs.</i>					
1	Pancreas + heart.	4 $\frac{2}{3}$	7.8	8.0	7.2	7.9	Dextrose.
2	" + "	5	7.9	8.0	6.8	7.6	"
3	" + "	5	7.7	7.9	6.8	7.4	"
4	Heart.	4	7.9	8.0	6.8	7.6	"
5	"	4	8.0	8.1	6.8	7.5	"
6	"	4	8.1	8.2	6.9	7.7	"
7	"	4	8.2	8.3	7.4	7.9	Levulose.
8	"	4	8.0	8.1	7.4	7.9	"
9	"	4	7.9	8.0	7.35	7.9	"
10	"	4	8.0	8.1	7.4	7.9	"
11	Pancreas + heart.	5	7.9	8.1	7.5	7.8	"
12	" + "	5	8.0	8.1	7.45	8.0	"
13	" + "	5	7.9	8.1	7.3	7.9	" - "
14	Heart.	4	8.0	8.1	7.5	7.9	Dextrose + levulose.
15	"	4	8.1	8.2	7.8	7.9	Dextrose + levulose.
16	"	4	8.1	8.2	7.5	7.8	Dextrose + levulose.
17	Pancreas + heart.	5	8.0	8.2	7.5	7.9	Dextrose + levulose.
18	" + "	5	8.0	8.15	7.4	7.9	Dextrose + levulose.
19	" + "	5	8.0	8.1	7.7	8.1	Dextrose + levulose.
20	" + "	5	8.0	8.2	7.55	7.9	Dextrose + levulose.
21	Pancreas + non-beating heart (control).	5	8.0	8.1	7.45	8.05	Dextrose + levulose.
22	Pancreas.	1 $\frac{1}{2}$	8.0	8.1	7.8	8.0	Dextrose.
23	"	4	8.1	8.2	7.8	8.0	Dextrose + levulose.
24	"	5	8.0	8.1	7.9	8.0	Dextrose.

*Controls.*—The following controls were used:

(a) In each experiment a sample of the original Locke's solution was subjected to the same procedure as the perfusate except for the actual circulation through the tissues. In this respect only the sample

TABLE II.

*Effect of the Locke's Solutions Used on the Optical Rotation of Dextrose and Levulose.*

No. of experiments averaged.	Sugar in the Locke's solution.	Method of treatment.		Duration of experiment.	Optical rotation with 4 dm. tube.					
					At beginning of period.		At end of period.		Change in rotation.	
						per cent		per cent		per cent
4	Dextrose.	Incubation, 3 experiments. Perfusion, 1 experiment.	Maximum.	24	+1.105°	0.526	+1.104°	0.525	+0.014°	0.0066
			Minimum.	4	+0.408°	0.194	+0.406°	0.193	−0.001°	0.0009
			Average.	9	+0.784°	0.373	+0.786°	0.374	+0.007°	0.0033
3	Levulose.	Incubation, 2 experiments. Perfusion, 1 experiment.	Maximum.	24	−1.572°	0.431	−1.572°	0.431	+0.004°	0.0019
			Minimum.	4	−1.313°	0.360	−1.310°	0.359	0.000°	0.0000
			Average.	10.6	−1.411°	0.387	−1.412°	0.387	+0.001°	0.0005
2	Dextrose + levulose.	Incubation, 2 experiments.	Maximum.	4	−0.792°		−0.792°		−0.006°	
			Minimum.	2	−0.726°		−0.720°		0.000°	
			Average.	3	−0.759°		−0.756°		−0.003°	

Average change in rotation in nine experiments in average of 7.5 hours = + 0.0016°.

of Locke's solution differed from the perfusate. This gave a fair basis for comparing the Locke's solutions and the perfusates.

(b) In a number of those experiments in which the pancreas was perfused alone, the unperfused splenic portion was macerated and ground aseptically with sand and about the same relative amount of Locke's solution as was perfused through the main portion of the

pancreas. This controlled the question whether an extract of the pancreas is similar in its effect to a perfusate.

(c) As enzyme action in general is sensitive to hydrogen ion concentration, the reactions of the Locke's solutions and perfusates were measured, in the majority of the experiments, by the colorimetric method of Levy, Rowntree, and Marriott (3). In Table I are given examples of the variations which occurred. While there was a certain variation both before and after removal of carbon dioxide, yet this variation was practically within the normal body limits and no effects were obtained which would indicate that a relation existed between the reaction of the perfusate and the experimental results.

(d) In Table II are given the results of nine experiments which show that when specimens of Locke's solution similar to those used in the perfusion experiments were incubated or perfused in the apparatus for varying periods of time, there is no essential variation in the optical rotation when either dextrose, levulose, or a mixture of the two is present. In the nine experiments with an average duration of 7.5 hours there was a maximum change in the rotation of  $0.014^{\circ}$  and a minimum change of  $0.000^{\circ}$ . The average change was an increased positive rotation of  $0.0016^{\circ}$ . These measurements were made in the 4 dm. tube used throughout this work.

## RESULTS.

### *Pancreas Perfusions.*

Eleven experiments were carried out in which a pancreas was perfused with an oxygenated Locke's solution containing dextrose. The final perfusates consistently showed a diminished optical rotation as compared with the Locke's solutions, but no change in the amount of reduction. The perfusates in four experiments were then hydrolyzed with the consistent result, also, that there was a partial restoration of the rotation to the original level, but no change in the reducing properties. These changes were considerably greater than any possible error in the polariscopic or reducing methods, and were controlled not only by the samples of Locke's solution subjected to an analogous procedure, but also, in five experiments, by the effect of extracts of a portion of the same pancreas on the same original

Locke's solution. The results are summarized in Table III and charted in Text-fig. 1. In Text-fig. 2 an experiment is given in which successive readings were made showing the curve of diminution in the optical rotation of a perfusate containing approximately 0.4 per cent of dextrose.

The question then arose: Was this phenomenon dependent upon a direct contact of the dextrose with the pancreatic tissue? Former experiments had shown that the rapid utilization of dextrose by the heart took place if dextrose was merely added to a pancreatic perfusate before being fed to the heart. In the three experiments shown in Table III and Text-fig. 1, the pancreas was first perfused with a Locke's solution containing no dextrose, and then dextrose was added to the perfusate and the solution allowed to incubate. As illustrated in Text-fig. 1, the change in rotation per unit of time was almost identical with the change which occurs when the pancreas is directly perfused with dextrose. This would indicate, therefore, that direct contact between the dextrose and pancreatic tissue is not necessary but that the change can be brought about by some substance washed out of the perfused gland.

Is this effect specific for dextrose in any way analogous to the specificity of most enzyme actions? Levulose was selected as a sugar for comparison. In six experiments, as shown in Table III and Text-fig. 1, the pancreas was perfused for an average period of 3 hours. In no experiment was there the least detectable change in the optical rotation or reducing properties of the sugar beyond the limits of experimental error. Hydrolysis of the final perfusates gave practically no change in the optical rotation or reduction. In four experiments, also, a pancreatic extract showed no effect. It seemed evident, therefore, that the action of the pancreas is specific for dextrose.

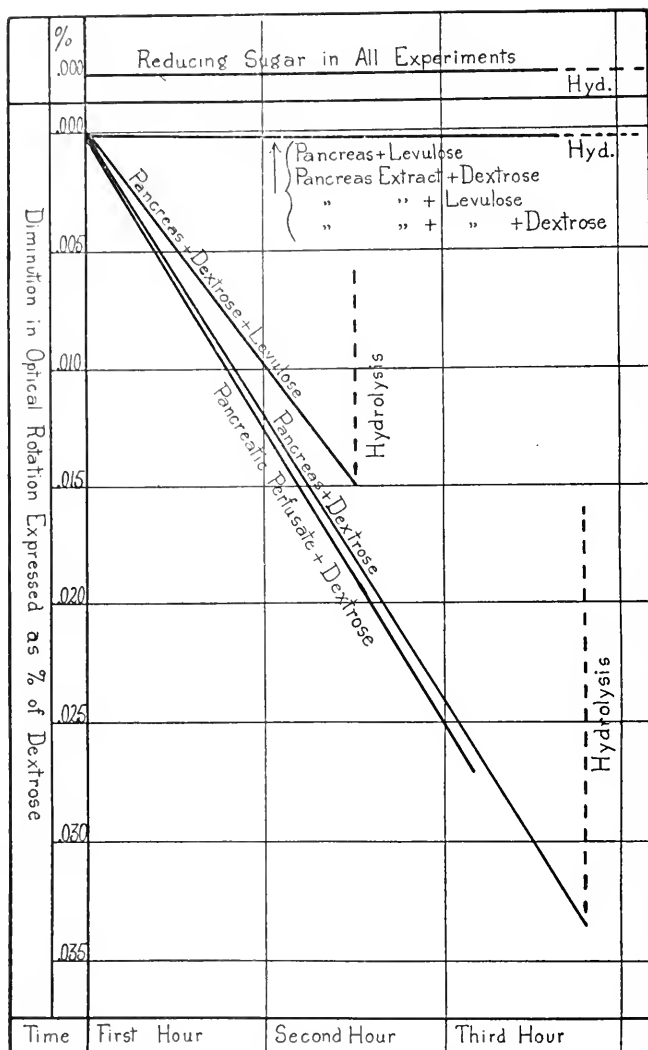
A further method for testing this specificity suggested itself. If a Locke's solution containing dextrose and levulose is used, there is a certain initial balance of rotation between the two sugars. If the effect of the pancreas is specific in diminishing the positive rotation of a dextrose solution, then, by selecting the dextrose from a mixture of the two sugars, there should be a shifting of the balance toward the negative side. This is what occurred in six experiments which were successfully carried out. The negative rotation was always

TABLE III.

*Experiments Showing the Effect of the Perfused Pancreas and of Pancreas Extracts on the Reducing Properties and Optical Rotations of Dextrose and Levulose.*

Optical rotation with 4 dm. tube.															Bacteria per cc. in final perfusate.		
No. of experiments averaged.	Sugar used.	Type of perfusion.	Duration of perfusion.	Before hydrolysis.					After hydrolysis.					Difference in reducing sugar between Locke's solution and perfusate.			
				Locke's solution.			Final perfusate.		No. of experiments hydrolyzed.	Change in optical rotation estimated as dextrose.			Locke's solution.			Final perfusate.	
				per cent	per cent	per cent	per cent	per cent		per cent	per cent	per cent				per cent	per cent
11	Dextrose.	Pancreas.	Maximum.	5	+0.880°	0.419	+0.838°	0.399	+0.002°	+0.0009	+0.046°	+0.022	0.002	4,000			
			Minimum.	0.6	+0.408°	0.194	+0.344°	0.163	0.000°	0.000	+0.016°	+0.007	0.000	2			
			Average.	2.8	+0.775°	0.369	+0.707°	0.336	+0.001°	+0.0004	+0.036°	+0.017	0.0007	700			
6	Levulose.	Pancreas.	Maximum.	4	-1.404°	0.386	-1.402°	0.385	0.000°	0.000	0.000°	0.000	0.000	1,800			
			Minimum.	1	-0.678°	0.186	-0.675°	0.185	0.000°	0.000	0.000°	0.000	0.000	6			
			Average.	3	-1.200°	0.329	-1.199°	0.329	0.000°	0.000	0.000°	0.000	0.000	1,000			
6	Dextrose + levulose.	Pancreas.	Maximum.	4	-0.292°	—	-0.324°	—	+0.002°	+0.0009	+0.036°	+0.017	0.001	900			
			Minimum.	1	-0.182°	—	-0.258°	—	0.000°	0.000	+0.020°	+0.009	0.000	3			
			Average.	1.5	-0.267°	—	-0.300°	—	+0.0005°	+0.0002	+0.019°	+0.009	0.0002	200			
5	Dextrose.	Pancreas extract.	Maximum.	4	+1.104°	0.525	+1.104°	0.525					0.000	800,000			
			Minimum.	2	+0.408°	0.194	+0.408°	0.194						0.000	240		
			Average.	3.6	+0.681°	0.324	+0.680°	0.323						0.000	268,000		

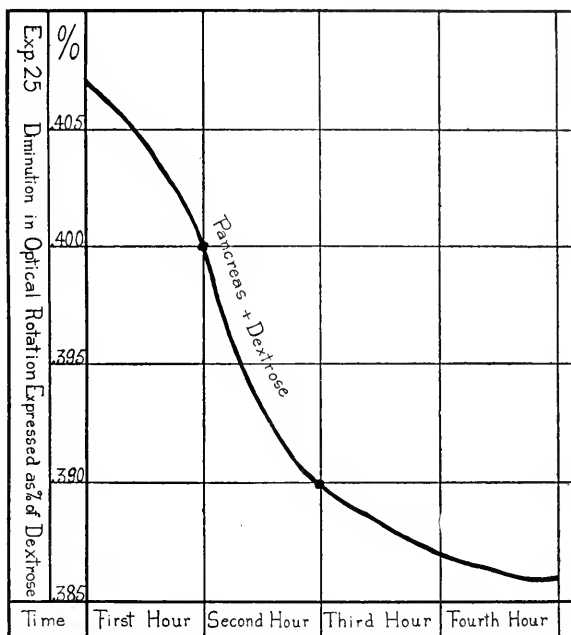
4	Levulose.	Pancreas extract.	Maximum. Minimum. Average.	4 4 4	-1.574° -0.678° -1.145°	0.432 0.186 0.314	-1.574° -0.676° -1.144°	0.432 0.185 0.314	0.0003						0.001 0.000 0.0005	3,000 200 1,700
2	Dextrose + levulose.	Pancreas extract.	Maximum. Minimum. Average.	4 2 3	-0.792° -0.720° -0.756°	— — —	-0.792° -0.720° -0.756°	— — —	0.000						0.001 0.000 0.0005	250 36 150
3	Dextrose.	Pancreas perfusate.	Maximum. Minimum. Average.	4 1.5 3.2	+1.106° +0.380° +0.721°	0.526 0.181 0.343	+1.050° +0.350° +0.665°	0.500 0.166 0.316	0.027						0.001 0.000 0.0007	3,100 2,000 2,600
4	Dextrose + levulose.	Spleen.	Maximum. Minimum. Average.	1 0.75 0.88	-0.314° -0.292° -0.300°	— — —	-0.314° -0.292° -0.300°	— — —	0.000	4	+0.004° -0.014° -0.014°	+0.0019 -0.006 -0.006	-0.014° 0.000° -0.008°	-0.006 0.000 -0.004	0.002 0.000 0.0007	140 0 56



TEXT-FIG. 1. Chart of Table III representing forty-one experiments. The chart shows that when the pancreas was perfused with a Locke's solution containing dextrose there was no change in the reducing properties of the perfusate but a distinct diminution in the optical rotation. This also occurred if dextrose was added to a sugarless perfusate after removal of the pancreas from the apparatus. A mixture of dextrose and levulose perfused through the pancreas gave an increased negative rotation indicating a diminution of the dextrose effect. On hydrolysis of these perfusates there was a partial restoration of the optical rotation. These changes did not occur if the pancreas was perfused with levulose nor did a fresh pancreatic extract have a similar effect on either sugar.



increased as shown in Table III and Text-fig. 1. The change in rotation estimated as dextrose per unit of time was only slightly less than if dextrose alone had been used. Hydrolysis gave a distinct restoration of the original balance. In no instance was there any variation in the reducing properties of the perfusates or Locke's solutions beyond the limits of experimental error.



TEXT-FIG. 2. Experiment 25. Curve showing the rate of diminution in optical rotation of a Locke's solution containing approximately 0.4 per cent of dextrose when perfused 4 hours through a pancreas. The reducing sugar remained unchanged.

These experiments were controlled by the action of pancreas extracts and by four experiments in which the spleen was perfused with a Locke's solution containing dextrose and levulose. In neither case was there any change, either in the optical rotation or in the reducing properties. With mixtures of dextrose and levulose, the conclusion would seem to be that the pancreas has a specific action on dextrose, an action which is not given by the perfused spleen, or by a pancreatic extract.

*Heart-Pancreas Perfusions.*

It was obvious from previous experiments with dextrose that a characteristic difference exists between a heart perfusate and one which has passed through both the heart and pancreas. Hydrolysis of a heart-pancreas perfusate gives an increase in reducing sugar, while a heart perfusate gives no such increase. The hypothesis suggested to explain this fact was that the heart and pancreas together brought about a condensation of the dextrose to some non-reducing form of polysaccharide.

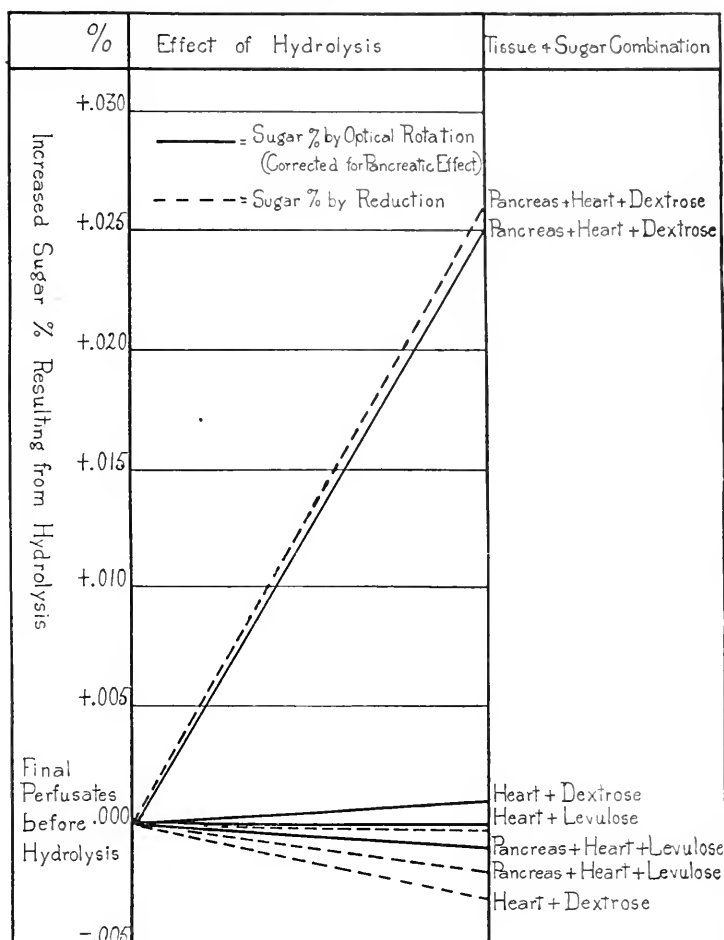
TABLE IV.

*Experiments on the Interrelation and Specificity of the Heart and Pancreas in Their Action on Dextrose and Levulose.*

No. of experiments averaged.	Sugar used.	Tissues perfused.		Final perfusate. Optical rotation with 4 dm. tube.					Final perfusate; change in reducing sugar on hydrolysis.
				Before hydrolysis.		After hydrolysis.		Change due to pancreas and heart, corrected.*	
					per cent		per cent	per cent	per cent
3	Dextrose.	Heart.	Maximum.	+0.648°	0.308	+0.650°	0.309		
			Minimum.	+0.532°	0.253	+0.534°	0.254		
			Average.	+0.591°	0.281	+0.592°	0.282		-0.0030
3	Levulose.	Heart.	Maximum.	-1.304°	0.358	-1.306°	0.359		
			Minimum.	-1.100°	0.302	-1.100°	0.302		
			Average.	-1.169°	0.321	-1.169°	0.321		0.0000
3	Dextrose.	Pancreas + heart.	Maximum.	+0.654°	0.311	+0.730°	0.347		
			Minimum.	+0.486°	0.231	+0.544°	0.259		
			Average.	+0.576°	0.274	+0.641°	0.305	+0.0250	+0.0260
3	Levulose.	Pancreas + heart.	Maximum.	-1.194°	0.328	-1.188°	0.326		
			Minimum.	-0.966°	0.263	-0.964°	0.262		
			Average.	-1.116°	0.306	-1.113°	0.305	-0.0004	-0.0020

The average amount of perfusate in each experiment was 252 cc.; the duration of each experiment, 4 hours. Bacteria per cc. in final perfusates: maximum, 640; minimum, 5; average, 122.

\* Corrected for the preliminary change in optical rotation due to the pancreas alone.



TEXT-FIG. 3. Chart of Table IV. The final perfusates from twelve experiments of different types were hydrolyzed and the reducing properties and optical rotations of the perfusates determined before and after hydrolysis. The percentage increase or decrease in sugar by these two independent methods is represented in this chart. The tissue and sugar combinations are indicated to the right. Correction is made for the previous independent effect of the pancreas on dextrose.

The experiments show the specificity of the heart-pancreas combination in condensing dextrose to a non-reducing substance with an optical rotation less than that of dextrose. This does not occur with the heart alone when perfused with dextrose or with any combination of the heart and pancreas with levulose.

The results of the twelve perfusions which were carried out are summarized in Table IV and shown graphically in Text-fig. 3. In these experiments the reducing properties and optical rotations of the final perfusates were determined before and after hydrolysis. If the optical rotation of a perfusate were altered by hydrolysis it would indicate that some new substance had been formed and whether the optical rotation of this substance were greater or less than the original sugar. A change in the optical rotation and an increase in the amount of reduction on hydrolysis would show that this substance had probably undergone a dissociative change.

The experiments clearly indicate that it is only with the heart-pancreas combinations that dextrose is transformed to a non-reducing substance which has an optical rotation less than that of dextrose. This transformation did not occur when the heart was perfused alone with dextrose nor did it occur with any combination of the heart and pancreas with levulose.

A clear demonstration of this specific action of the pancreas and heart on dextrose was shown in the twelve experiments given in Table V and Text-fig. 4. In these experiments the pancreas, the spleen, or the kidneys were first perfused, and their perfusates compared in their ability to enable the heart to select dextrose from a mixture of dextrose and levulose. A displacement of the balance in optical rotation of a perfusate containing approximately equal parts of these sugars would demonstrate which sugar had been changed or used most rapidly. Hydrolysis of the final perfusates would, further, indicate, by the extent to which the optical rotation was restored, the amount of change which had taken place.

The results of these experiments were definite. When the heart was perfused alone with a Locke's solution containing dextrose and levulose, there was a distinct utilization of the sugars as shown by the change in reduction. The optical rotations of the final perfusates, however, either were unchanged or were slightly increased on the negative side. This gave definite proof that both sugars had been used either in approximately equal amounts or that there had been a slightly greater utilization of dextrose over levulose. If the spleen was first perfused and the perfusate subsequently passed through the heart, the selective action of the heart for dextrose was practically

the same as with the heart alone. Finally, the kidneys were perfused and gave a certain removal of levulose but subsequent perfusion of the fluid through the heart gave a shift in the optical rotation which, once more, was only slightly greater than with the heart alone.

TABLE V.

*Experiments on the Specificity of the Pancreas in Enabling the Heart to Select Dextrose from a Mixture of Dextrose and Levulose.*

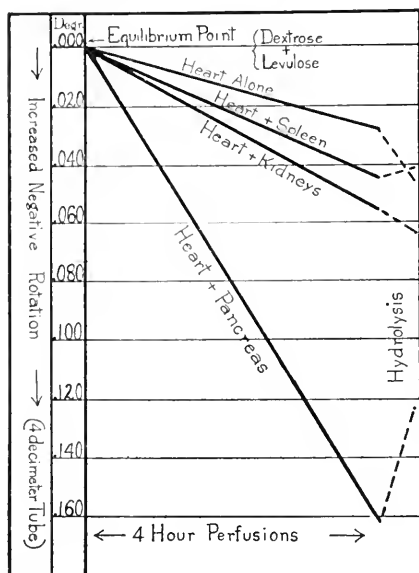
No. of experiments averaged.	Tissues perfused.		Optical rotation with 4 dm. tube.					
			Locke's solution.		Final perfusate.			
			Before hydrolysis.	After hydrolysis.	Before hydrolysis.	Increased negative rotation, corrected.*	After hydrolysis.	Change in rotation after hydrolysis, corrected.*
4	Heart.	Maximum.	-0.308°	-0.306°	-0.350°			Increased negative 0.013°
		Minimum.	-0.280°	-0.264°	-0.286°			
		Average.	-0.295°	-0.285°	-0.323°		-0.326°	
4	Pancreas + heart.	Maximum.	-0.292°	-0.286°	-0.520°			Diminished negative 0.027°
		Minimum.	-0.280°	-0.274°	-0.428°			
		Average.	-0.286°	-0.280°	-0.460°	0.162°	-0.415°	
2	Kidneys + heart.	Maximum.	-0.250°	-0.242°	-0.300°			Increased negative 0.003°
		Minimum.	-0.119°	-0.121°	-0.190°			
		Average.	-0.184°	-0.181°	-0.245°	0.056°	-0.250°	
2	Spleen + heart.	Maximum.	-0.314°	-0.300°	-0.368°			Increased negative 0.004°
		Minimum.	-0.292°	-0.290°	-0.328°			
		Average.	-0.303°	-0.295°	-0.348°	0.045°	-0.344°	

The average amount of perfusate in each experiment was 250 cc.; the duration of each experiment, 4 hours. Bacteria per cc. in final perfusates: maximum, 880; minimum, 0; average, 183.

\* Corrected for rotation due to pancreas, kidneys, or spleen alone.

In contrast to these experiments were those in which the pancreas was first perfused and the perfusate subsequently circulated through the heart. Instead of an optical rotation which varied either not at all or only moderately from an equilibrium between the two sugars, there was a striking shift to the left even after correcting for

the preliminary effect of the pancreas. Hydrolysis of the final perfusate, also, diminished the negative rotation to some extent, while



TEXT-FIG. 4. Chart of Table V. Twelve experiments in which the spleen, kidneys, and pancreas were first perfused and their perfusates compared in their ability to enable the heart to select dextrose from a mixture of dextrose and levulose. Displacement of the balance in optical rotation of a perfusate containing equal parts of these sugars would indicate which sugar had been changed or used most rapidly, while hydrolysis of the final perfusates would indicate, by the extent to which the optical rotation was restored, the amount of condensation and of utilization. Correction was made in each experiment for any preliminary effect of the spleen, kidneys, or pancreas.

The experiments indicate that the heart, or the heart plus spleen or plus kidneys over a 4 hour period of perfusion, uses relatively a little more dextrose than levulose. When, however, the heart and pancreas are combined the relative utilization of dextrose by the heart is greatly increased and subsequent hydrolysis of the perfusates restores the optical rotation to some extent, which does not occur when the spleen or kidneys are combined with the heart.

in the previous experiments where the heart had been perfused alone or with the kidneys or spleen, hydrolysis of the final perfusates had slightly increased the negative rotation.

*Osazones.*

A further possible method of demonstrating the above changes was available in the melting points of the osazones which could be obtained at different stages in various types of experiments when dextrose was used. The following technique was observed: 10. cc. specimens were taken, both of the original Locke's solution and of the protein-free perfusate. They contained the same reagents and differed in their previous and subsequent treatment only in the actual perfusion. These samples were carefully neutralized with sodium carbonate, then made equally acid with acetic acid. To each specimen were added 1.5 gm. portions of a pulverized mixture of two

TABLE VI.  
*Melting Point of Osazones, Corrected.*

Experiment No.	Locke's solution.		Pancreas perfusate.		Final heart perfusate.	
	Before hydrolysis.	After hydrolysis.	Before hydrolysis.	After hydrolysis.	Before hydrolysis.	After hydrolysis.
	°C.	°C.	°C.	°C.	°C.	°C.
16 (heart alone.)	204.4				204.9	205.4
17	205.59	204.09	201.58	204.09	200.57	203.58
18	204.05	205.02	203.55	206.52	202.62	206.02
19		205.02	201.51	205.52	200.50	205.52

parts of sodium acetate and one part of phenylhydrazine hydrochloride. As soon as these reagents were dissolved the specimens were filtered into test-tubes and placed in a boiling water bath for an hour. After cooling, the osazones were filtered off, washed with distilled water, and recrystallized from alcohol. The melting points were determined in capillary glass tubes by the usual method. Not the point at which the osazones sintered but the actual melting points were taken and the proper thermometer correction was made.

Table VI gives the results of twenty such determinations. The results show no essential diminution or increase in the melting points of the osazones yielded by the samples of original Locke's solution either before or after hydrolysis. Perfusion of a heart alone did not

materially lower the melting points of the osazones obtained. On the other hand, the osazones from the pancreatic and the heart-pancreas perfusates gave a distinct lowering of the melting points, while the same perfusates hydrolyzed gave osazones with melting points again approximately similar to those obtained from the Locke's solutions. Thus this osazone effect occurred only under those conditions in which the evidence from the optical rotation indicated a condensation of dextrose.

It is obvious that the osazones obtained in this way would consist of a mixture of glucosazone and the new osazones formed, and that the lowered melting points of the mixtures would be dependent upon the fact that the new osazones had a lowered melting point. The amounts of osazones available were so small relatively that no effort was made to isolate them in order to determine their independent melting points.

The only evidence from the osazones as to any qualitative or quantitative difference between the polymerization taking place in the pancreatic and heart-pancreas perfusions was in the slightly lower melting points of the latter. The general conclusion would seem to be that both the pancreas and the pancreas and heart bring about the formation of a substance or substances which yield osazones with lower melting points than glucosazone.

#### *Bromine Analyses.*

A possible method for studying the specificity of the heart and pancreas in their selection of dextrose was available in the property which bromine possesses of destroying dextrose but causing little if any change in levulose. Dextrose could thus be eliminated from a mixture with levulose and the amount of remaining levulose determined with the polariscope. The technique consisted simply in adding an excess of bromine to the acidified, hydrolyzed, protein-free specimens of fluid in glass-stoppered bottles, and, after thorough shaking, allowing them to stand for 7 days at 37°C. By using the hydrolyzed specimens the factor of any preliminary condensation was eliminated. All experiments were controlled by duplicate samples of the original Locke's solutions which had passed through an analo-



gous procedure. The bromine was removed by bubbling air through the fluids. Polariscopic readings then showed approximately the amounts of levulose which had been present in the original mixtures. The results of eleven experiments are collected in Table VII and the significant findings illustrated in Text-fig. 5. The first six experiments show that, by the method used, with dextrose alone there was always a small residue of positive rotation. In two experiments when a mixture of dextrose and levulose of approximately

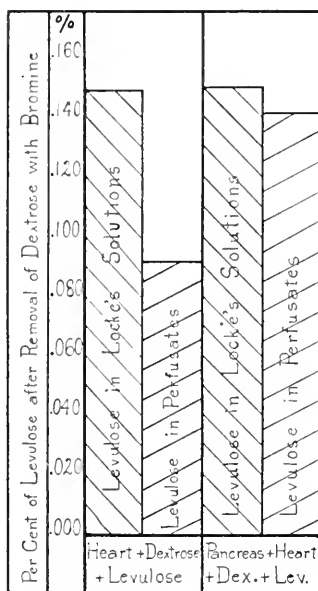
TABLE VII.

*Experiments Showing the Results of Bromine Treatment of the Final Perfusates.*

Experiment No.	Tissues perfused.	Sugar used.	After bromine treatment.			
			Locke's solution.		Perfusate.	
			Optical rotation with 4 dm. tube.	Sugar.	Optical rotation with 4 dm. tube.	Sugar.
				<i>per cent</i>		<i>per cent</i>
26	Heart.	Dextrose.	+0.10°	0.047	+0.10°	0.047
27	"	"	+0.04°	0.018	+0.09°	0.042
28	"	"	+0.10°	0.047	+0.09°	0.042
29	Pancreas.	"	+0.05°	0.023	+0.10°	0.047
30	"	"	+0.08°	0.038	+0.12°	0.057
3	"	"	+0.03°	0.014	+0.05°	0.023
16	Heart.	" + levulose.	-0.508°	0.139	-0.214°	0.059
31	"	" + "	-0.578°	0.159	-0.454°	0.124
17	Pancreas + heart.	" + "	-0.550°	0.151	-0.526°	0.144
18	" + "	" + "	-0.602°	0.165	-0.524°	0.143
19	" + "	" + "	-0.488°	0.134	-0.502°	0.137

constant composition was perfused through the heart, the amount of levulose, after bromine treatment, was considerably diminished in comparison with the original Locke's solution, similarly treated. This meant, in the two experiments conducted, that a certain proportion of levulose had been used by the heart. When, however, a pancreas perfusate with the same composition was fed to a heart, the amount of levulose was essentially undiminished, though the utilization of sugar had occurred to the usual extent. The utilization of sugar, therefore, when the heart and pancreas were combined,

must have been from the dextrose portion of the mixture. These results agree with the previous findings that the heart and pancreas have a specific action on dextrose.



TEXT-FIG. 5. The results in five experiments of treating the final perfusates with bromine for 7 days. A solution containing dextrose alone loses practically all of its sugar under this bromine treatment, while levulose is practically unaltered. When a heart is perfused with a solution containing practically equal parts of dextrose and levulose there is a considerable disappearance of levulose from the final perfusate as compared with the original Locke's solution. When the heart and pancreas, however, are combined in their effect, in spite of the increased sugar consumption there is practically no utilization of levulose.

#### DISCUSSION.

The following explanation seems to be most consistent with these facts and with those brought out in my previous work. The pancreas supplies to a perfusate some enzyme or enzymes which have a specific action on dextrose as compared with levulose, changing a certain portion of the dextrose to a simple form of polysaccharide. This polysaccharide has both a lower optical rotation and a lower

melting point of its osazone than dextrose. I should regard this sugar complex as either relatively unstable, being hydrolyzed during the reduction determination, or as having the same reducing properties as dextrose. When, however, the pancreatic perfusate is circulated through a living heart the optical rotation not only continues to diminish but a new change occurs. The reducing properties of the perfusate are now altered. Thus the effect of the heart seems to be the production of a change additional to that caused by the pancreas. The most probable explanation would seem to be a further polymerization of the sugar in the presence of the living heart to a more stable form with a diminished power of reduction. Neither of these apparent syntheses is brought about by an extract of the ground pancreas, by the perfusion of the spleen, the kidneys, or the heart alone, or by a combined perfusion of the pancreas and heart with levulose. As to the nature of these probable polysaccharides formed from dextrose, nothing definite can be stated, but the facts described tend to eliminate certain known sugars from consideration. Thus, because the optical rotations of the perfusates are diminished, maltose, isomaltose, saccharose, and glycogen cannot be considered, for each gives a greater positive rotation than dextrose. Lactose is also eliminated, for its rotation is identical with that of dextrose.

The supposition that synthetic changes are brought about by the pancreas or by the combined influence of the heart and pancreas, is in accord with numerous examples of the synthetic actions of enzymes which have been experimentally demonstrated by other observers. Thus, Hill (4) showed that maltase could synthesize a certain proportion of dextrose to what he thought was maltose. Lombroso (5) brought about a synthesis of fats by the action of pancreatic lipase. Taylor (6) obtained a protamine from its cleavage products by the action of trypsin. In my experiments some such synthetic action seems to be a more likely explanation of the results and one better in accord with our general knowledge of enzymes and sugars than the alternative suggestion of some essential change in the configuration of the dextrose molecule. The facts that osazones with lower melting points are obtained, and that the optical rotations of the perfusates and the melting points of the osazones can be reestablished to some extent by hydrolysis with weak acid, would be strong evidence that synthesis had occurred.

In this connection it is suggestive to note that the presence of more complex sugars than dextrose has been apparently demonstrated both in the blood and body tissues by various workers. Thus Pavy (7) and Lépine (8) by hydrolyzing blood increased its reducing properties. In a recent publication Palmer (9) has clearly shown that extracts of heart or skeletal muscle give a similar increase of reduction on hydrolysis. Again, a number of workers have obtained osazones with lower melting points than glucosazone from blood and muscle extracts. Panormoff (10) secured an osazone with a low melting point from extracts of dog muscle. From the blood Pavy and Siau (11) obtained an osazone which melted at 153°C., and suggested its identity with Fischer's "isomaltose." Osborne and Zobel (12) by more careful work concluded that this substance was maltose. Levene and Meyer (13), by combining muscle and pancreas extracts in the presence of relatively concentrated solutions of dextrose, isolated a biosazone which melted at about 200°C. The possible association of these facts with the present work is of interest.

The evidence previously advanced for the enzyme character of the substance obtained from the perfused pancreas is corroborated further in these experiments by the specificity of its action on dextrose as compared to levulose, and by the fact that the changes do not proceed to completion. Within recent years the specificity of enzyme action has received many proofs and in general it has been found that when an enzyme action is selective between two simple sugars, the selection is dependent upon the configuration of the sugar molecule rather than upon the presence of ketone or aldehyde radicals. It would seem possible that the selective action of the pancreas for the normal body sugar, dextrose, as compared to levulose also depends upon the molecular configuration rather than upon the fact that dextrose is an aldehyde and levulose a ketone sugar.

From the experiments here reported, it cannot be determined whether the specific sensitization of the heart for dextrose is dependent merely upon the preliminary change in the sugar produced by the pancreas. It seems probable that the action of the pancreatic enzyme does not cease with a simple polymerization but that it initiates a number of changes, the subsequent steps of which are dependent upon the interrelation of the enzyme with the living heart. As far as the heart alone is concerned there is a similarity in its action to that of an eviscerated diabetic animal as described by Macleod and Pearce (14). In both there is a certain apparent utilization

of dextrose, but for the isolated heart, at least, this utilization is non-specific, for it occurs almost equally well with levulose. In both, however, there is a certain fundamental incompleteness in the power to utilize sugar. With the heart, by supplying a pancreatic perfusate, a highly specific relation is established between it and the circulating dextrose which not only causes an immediate and rapid utilization of dextrose but also brings about a certain condensation of dextrose which can be initiated by the pancreas alone. This specific interrelation of the pancreas and heart to dextrose suggests that in normal sugar metabolism this pancreatic enzyme, or enzymes, may be necessary; that certain stages of synthesis and polymerization may be of importance as intermediate steps in carbohydrate utilization; and that when there is an insufficiency of the pancreatic function, though the body tissues are supplied with an abundance of dextrose which can be burned to a certain extent, yet the essential steps by which dextrose is prepared for normal utilization cannot take place.

#### SUMMARY.

When the pancreas of a dog is perfused aseptically with a Locke's solution containing dextrose in physiological concentrations, the optical rotation of the perfusate is diminished, but its reducing power is unaltered. This change also occurs if dextrose is added to a sugar-free pancreatic perfusate and the mixture incubated. These perfusates yield osazones with lower melting points than glucosazone, but when the perfusates are hydrolyzed with weak acid their optical rotations and the melting points of their osazones are increased. These changes do not occur with levulose, or with an extract of the pancreas and dextrose. When the heart, spleen, or kidneys are perfused with dextrose solutions hydrolysis of the perfusates does not increase their optical rotation or power of reduction.

When a pancreatic perfusate containing dextrose is circulated through a living heart not only do the above changes take place but, in addition, the reducing properties of the perfusate are altered. Hydrolysis of such a perfusate increases its reducing power, its optical rotation, and the melting point of its osazone. A heart does not cause this effect either alone or when perfused together with the

spleen or kidneys. Levulose perfused through the heart and pancreas is unchanged.

These phenomena are believed to be due to an enzyme or enzymes obtained from the perfused pancreas. The changes in optical rotation, in reduction, and in the osazones are accounted for by different degrees of dextrose condensation. While the living heart can destroy both dextrose and levulose to some extent, the experimental results suggest that the enzyme or enzymes derived from the perfused pancreas have a specific action on dextrose and are responsible for certain essential steps by which dextrose is prepared for normal utilization.

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# CARRIAGE OF THE VIRUS OF POLIOMYELITIS, WITH SUBSEQUENT DEVELOPMENT OF THE INFECTION.

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PLATES 55 AND 56.

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The solution of the problem of the mode of infection in poliomyelitis has been attempted in various ways, with results which have led to the conclusion that the microbic cause is conveyed from one individual to another by personal contact. This belief is based upon clinical observation and experiment. Wickman first brought clinical proof, since supported by many independent observations, of the correctness of this generalization; and Flexner and Lewis, and later Kling and Pettersson, provided the experimental demonstration of its adequacy.

However, a considerable number of physicians and others still refuse to accept this explanation. They hold that the mode of infection remains undiscovered, or they account for it through some variety of insect transmission, also undetected. In recognition of the skepticism still prevailing, we have been led to describe in detail the experimental demonstrations of the carriage by healthy persons of the virus of poliomyelitis, to which may now be added our own successful inoculations. Our results include the demonstration, recorded for the first time, that a proved carrier of the virus may come down with acute poliomyelitis. This observation should serve to strengthen the position of those who accept as established the personal communication of the microbic cause, or virus, of the disease.

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*Previous Reports.*

• Wickman's<sup>1</sup> clinical studies may be said to have disseminated the view of the personal factor in the communication of the virus of poliomyelitis. He emphasized the occurrence and epidemiological importance of the non-paralytic or abortive cases, the first description of which is usually credited to him, and of healthy intermediaries, or bacillary carriers, who function as purveyors of the microbic agent. His study constituted a great step forward; but the first person to allude to non-paralytic cases of epidemic poliomyelitis is Caverly,<sup>2</sup> who records the occurrence of 6 cases among the total of 132 cases on which he based his report describing the Rutland epidemic of 1894.

Soon after Landsteiner and Popper's<sup>3</sup> experimental transmission of poliomyelitis, Flexner and Lewis<sup>4</sup> detected the virus in the nasopharyngeal mucous membrane of infected monkeys. This observation, soon confirmed by several independent bacteriologists, was followed by a study made by Kling, Pettersson, and Wernstedt<sup>5</sup> who injected into monkeys buccal washings from so called abortive cases and from healthy contacts. Their results were inconclusive, as the clinical condition produced was not typical of poliomyelitis, and the pathological changes described as present in the spinal cord were not characteristic of the disease. They explained the discrepancy by the supposition that the virus present in the abortive cases and healthy carriers was relatively avirulent. This view is repeated in their recent report<sup>6</sup> in which they describe an instance of healthy carriage of the highly active virus inducing paralysis and characteristic lesions. The first demonstration of the typical virus in the nasopharyngeal washings of healthy persons was, however, made by Flexner, Clark, and Fraser,<sup>7</sup> whose report follows in detail.

E. A., female, age 4 years and 4 months. The patient had been ill from Oct. 12 to 17, 1912. On the latter date she was admitted to the Hospital of The Rockefeller Institute for Medical Research, suffering from severe paralytic polio-

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<sup>1</sup> Wickman, I., Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

<sup>2</sup> In view of the importance which the non-paralytic cases have assumed in the epidemiology of poliomyelitis it is pertinent to quote Caverly, who states that paralysis occurred in 119 cases, 7 cases died before paralysis was detected, "and the remaining 6 had no paralysis, but all had a group of symptoms very common in the initial stage in those which were paralyzed, such as headache, fever, convulsions, or nausea, one or all" (*J. Am. Med. Assn.*, 1896, xxvi, 1).

<sup>3</sup> Landsteiner, K., and Popper, E., *Z. Immunitätsforsch., Orig.*, 1909, ii, 377.

<sup>4</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1140.

<sup>5</sup> Kling, C., Pettersson, A., and Wernstedt, W., *Communications Inst. méd. État à Stockholm*, 1912, iii, 5.

<sup>6</sup> Kling, C., and Pettersson, A., *Deutsch. med. Woch.*, 1914, xl, 320.

<sup>7</sup> Flexner, S., Clark, P. F., and Fraser, F. R., *J. Am. Med. Assn.*, 1913, lx, 201.



myelitis. She subsequently improved and was discharged. Oct. 28. The mother and father of the child were subjected to a nasopharyngeal irrigation with normal saline solution; about 150 cc. of washings were obtained. The fluid was shaken and passed through a Berkefeld filter; of the filtrate, 1.5 cc. were injected the same day into the sheath of each sciatic nerve and 140 cc. into the peritoneal cavity of a *Macacus cynomolgus* (Monkey A). Recovery from the anesthesia was prompt and the animal remained well until Nov. 11, when it was noted to be excited and to drag the right leg; the left leg was weak. Nov. 12. Right leg flaccid. A lumbar puncture yielded 2.5 cc. of fluid containing excess of white corpuscles. Nov. 13. The condition was unchanged; the animal was etherized. The organs generally were normal in appearance; the spinal cord was edematous. Microscopic examination of sections of the spinal cord, medulla, and interstitial ganglia revealed the characteristic lesions of poliomyelitis. The blood vessels and ground substance showed infiltrations with mononuclear cells; the motor nerve cells were degenerated and invaded by phagocytes.

Dec. 3. An emulsion of the glycerolated spinal cord and medulla was injected into each sciatic nerve and the peritoneal cavity of a *Macacus cynomolgus* (Monkey B) and a *Macacus rhesus* (Monkey C). Dec. 9. The *rhesus* monkey was noted to be excited. Dec. 10. Lumbar puncture yielded 3 cc. of turbid fluid containing excess of white cells. By Dec. 13, the legs were partially paralyzed; the animal was etherized. Microscopic sections of the spinal cord, medulla, and intervertebral ganglia showed typical infiltrative and degenerative lesions of poliomyelitis. The *cynomolgus* monkey became excited on Dec. 10, and on the 19th paralysis of the legs appeared. By Dec. 21 the arms and back were weak, and the paralysis was extending. Dec. 23. The animal was etherized. The general viscera appeared normal, but the spinal cord was both edematous and congested. The microscopic sections of the cord, medulla, and intervertebral ganglia showed typical infiltrative and degenerative lesions attended by neurophagocytosis. Subsequently the glycerolated specimens of the nervous organs of Monkeys B and C were used for inoculating still other monkeys, in which typical paralysis was induced.

The conclusion drawn by the authors from this demonstrative experiment was to the effect that the parents of E. A., neither of whom showed any symptoms of illness and who evidently were not suffering from poliomyelitis, harbored the virus of the disease in the nasopharynx. Hence the existence of the healthy carrier was thus established experimentally.

The next demonstrative experiment was supplied by Kling and Pettersson<sup>6</sup> who, in referring to their earlier failure to produce clinically and anatomically typical poliomyelitis with nasopharyngeal washings, attribute the failure to the injection of insufficient amounts of virus into the monkeys. They repeated the tests, using washings concentrated *in vacuo* with the Faust-Heim apparatus.

They started out by determining the heat lability of the active virus, and ascertained that a liter of fluid carrying an effective dose could be evaporated at

temperatures ranging from 35 to 38°C. to 200 cc. without losing its potency. They now obtained nasopharyngeal washings in amounts of 1 to 2 liters from healthy persons in contact with cases of acute poliomyelitis. In one instance in which the washings were taken from the healthy members of a family in which one member had recently died of acute poliomyelitis, the inoculation resulted successfully.

The patient was a male, age 41 years. The illness began on Sept. 10, the legs becoming paralyzed 2 days later. Death took place on the 4th day of illness from respiratory failure. The surviving members of the family consisted of the wife and three children ranging from 10 to 14 years, all remaining well. One day after the death of the father in a hospital, nasal washings were taken in distilled water from the surviving members of the family. The combined washings, amounting to 1 liter, were evaporated *in vacuo* to 75 cc., sodium chloride was added, and the mixture was filtered first through paper and then through a Berkefeld candle.

Sept. 20. 0.5 cc. of the filtrate was injected intracerebrally and 20 cc. were introduced into the peritoneal cavity of a *Macacus sinicus*. Oct. 2. The right leg and on the next day both legs and back were paralyzed, and death resulted. The microscopic sections of the spinal cord showed moderate perivascular and diffuse infiltration of the nervous tissue with mononuclear cells and neurophagocytosis. Oct. 3. A second *Macacus sinicus* was inoculated intracerebrally and intraperitoneally with an emulsion of the spinal cord of the first animal. On Oct. 13 the right leg and on the next day the left leg were paralyzed. Oct. 15. The animal was killed. Sections of the spinal cord showed typical infiltrative and degenerative lesions of poliomyelitis.

There can be no doubt, therefore, that in this family one or more healthy carriers of the active virus of poliomyelitis existed. That the result was not due entirely to the employment of concentrated washings is indicated by the failure to detect the virus in the washings obtained from the healthy associates of two other cases of acute poliomyelitis.

#### OBSERVATIONS.

In the two successful instances just reviewed, mixed washings were employed for inoculation. It is, therefore, impossible to state whether one or more of the healthy contacts of the cases of poliomyelitis were carriers. In the instance which we shall report the individuals were irrigated separately. The final result proved that more than one virus carrier was present, and it was demonstrated that such a healthy carrier may develop poliomyelitis. We may

therefore regard the chain of the mode of infection as now having been completed for the first time. The separate links may be defined as follows:

Case of acute poliomyelitis → contact carrier → second case.

A still further analysis would determine that through the contact carrier other carriers occur, among which a certain number of additional cases arise.

Poliomyelitis occurred in epidemic form in Washington County, Vermont, in the summer of 1917. From June 1 until September 1, 79 cases were recognized among the population of 45,000.

Carey P., male, age 16 years. The patient lived in the village of Waitsfield, 18 miles from Montpelier, where cases of poliomyelitis existed. No case of the disease had been discovered in Waitsfield. On June 2, 1917, he attended a ball game at Northfield where there were no cases, and returning home stopped in Montpelier for supper. Probably in the assembly at Northfield persons from the infected district were present. Until June 12 there were no symptoms of illness; on that day there was complaint of headache and pain in the back and legs. The patient vomited once. June 13. First seen by a physician who observed that the patient had fever, and treated him for a gastrointestinal upset. June 16. Extensive paralysis involving both legs, right triceps, intercostals, pectorals, and diaphragm. Lumbar puncture yielded clear fluid under pressure, containing 400 white cells per cmm. and excess of globulin. Death occurred on this date.

The family consisted of the father, age 59 years, mother, age 42, sister, Hazel, age 13, two brothers, Everett, age 10, and Dwight, age 7. The two younger brothers slept in the same bed, and in the same room with the elder brother Carey.

June 16. Everett and Hazel were given nasopharyngeal irrigation with distilled water, 60 cc. being obtained from the former and 100 cc. from the latter. 10 per cent of ether was added to each, and the fluids were sent at once to the laboratory. One of us had previously determined that ether inhibits bacterial development without injuring the poliomyelitic virus. The washings were treated separately as follows: Glass beads were added and they were shaken mechanically for  $2\frac{1}{2}$  hours. They were then centrifuged at high speed for  $2\frac{1}{2}$  minutes, and the supernatant fluid was passed through a Berkefeld N candle and concentrated *in vacuo* by the method already described by us<sup>8</sup> at 35°C. to 2 cc. The entire concentrate was injected intra-

<sup>8</sup> Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

cerebrally into two *Macacus rhesus* monkeys (Monkey A (Everett) and Monkey B (Hazel)). The time elapsing between the collection and the injection of the washings was less than 6 hours.

We return briefly to the history of the two children. Everett had not been away from the village and was in usual health until June 13, the day after Carey fell ill. He also felt indisposed, showed a temperature of 102° F. and suffered from diarrhea, but did not vomit. However, he recovered quickly and subsequently on minute examination has shown no muscular weakness or abnormality of reflexes.

Hazel had not been away from Waitsfield. She had been entirely well at the time the washings were taken and remained well until June 21, at which time she complained of headache. She showed a temperature of 102° F. On June 22 her reflexes were exaggerated and stiffness of the back was present, but no muscular weakness was detected. Lumbar puncture was unsuccessful. The symptoms subsided gradually, but reexamination made on July 22 revealed partial paralysis of the left deltoid, right anterior tibial, and abdominal muscles. She had, therefore, suffered a mild attack of poliomyelitis.

*Monkey A.*—June 16, 1917. Inoculated. Remained well until June 29, when it was excitable, emitted staccato cries, and showed ruffled hair. The animal was noted to be clumsy in movement and unable to jump. June 30. Both legs were weak. July 4. The right leg was paralyzed and flaccid; the left leg and back were weak. The paralysis of the left leg and back, but not of the right leg, disappeared; the latter remained and contracture gradually set in. At the present time (Sept. 1) the contraction of the right leg is so marked that in moving about the animal does not touch the limb to the floor. On Aug. 8 blood was withdrawn for a neutralization test and at the same time an intracerebral inoculation was made with a large dose of virus proved active in another monkey; the result was negative. The animal, as is usually the case, having recovered from a recent infection, was resistant to reinoculation.

*Monkey B.*—June 17, 1917, 3 a.m. Injected intracerebrally with 1.5 cc. of the concentrated washings. Recovery from the anesthesia was immediate, and the first symptoms, consisting of excitability, ruffled hair, staccato cries, and partial paralysis of the right leg, were observed. June 26. The paralysis being stationary, the animal was etherized. The organs appeared normal to the naked eye. Microscopic sections revealed, however, marked typical lesions of poliomyelitis. They affected the spinal cord (Fig. 1), medulla (Fig. 2), and intervertebral ganglia (Figs. 3 and 4), and consisted of typical infiltration with mononuclear cells and nerve cell degeneration with phagocytosis.

*Monkey C.*—June 26, 1917. Injected intracerebrally under ether anesthesia with 2.5 cc. of a 20 per cent emulsion of spinal cord and medulla of Monkey B. July 7. The first symptoms were noted, consisting of ruffled hair and inclination of head to the left. July 8. The animal was ataxic and protected the right leg. July 9. Unable to jump; legs and back weak. July 10. Paralysis progressing. July 15. Etherized. The spinal cord showed typical focal lesions of poliomyelitis in which cicatrization was beginning.

These experiments leave no doubt that the washings, both from Everett and from Hazel, contained the virus of poliomyelitis. The instance of Hazel is of particular importance since in her case the virus was detected in washings taken 5 days before the first symptoms of what proved subsequently to be a mild attack of poliomyelitis set in. In other words, she was carrying the virus in her nasopharynx several days in advance of the appearance of any signs of illness. She constitutes, therefore, an example of a carrier of the virus developing poliomyelitis—the first one in which the demonstration has been proved experimentally.

The interpretation in the case of Everett is not so simple. When the virus was detected in his nasopharynx he had passed through a slight attack of illness, at about the same time with, and of about the same character as that of his brother Carey who died, but unattended by paralysis. The presumption is that Everett suffered from a non-paralytic or abortive attack of poliomyelitis. The detection of the virus in his case proves him not to have been a healthy, but a recovered carrier of the microbic cause of the disease.

The two children having been shown to be virus carriers, their nasopharyngeal secretions were tested by the method of Amoss and Taylor,<sup>8</sup> to determine whether they would neutralize an active poliomyelitic virus.

July 23, 1917. Washings with sterile water were taken from the children, and fractionally sterilized and mixed. To 15 cc. of the mixture were added 3.75 cc. of a Berkefeld filtrate of a 5 per cent stock glycerolated poliomyelitic spinal cord. After shaking, the combined fluids were permitted to remain at 37°C. for 24 hours. 1 cc. of the fluid was injected intracerebrally into a *Macacus rhesus*. No symptoms appeared until Aug. 4, when excitability, ataxia, paralysis of the right arm, and weakness of the back were noted. Aug. 8. Animal prostrate. Aug. 10. Died. The microscopic lesions were typical of poliomyelitis.

The mixed nasal washings failed, in this experiment, to neutralize the virus.

The youngest child, Dwight, age 7 years, was refractory and no washings were obtained from him on June 16 when they were taken from the other children. On June 18 he complained of being unwell. The symptoms were severe headache, stiffness of neck, exaggerated reflexes, but no diarrhea. Lumbar puncture yielded a fluid containing 500 white cells per cmm. and an excess of globulin. Immune poliomyelitic serum from recovered cases of the disease was administered intraspinally, intravenously, and subcutaneously: 24 cc. were given intraspinally, 30 cc. intravenously, and 39 cc. subcutaneously. Recovery was prompt, with a slight paralysis of the right anterior tibial muscle. Nasopharyngeal washings were, however, obtained on September 4, which after filtration and concentration were inoculated into a *Macacus rhesus* (Monkey D). The monkey remained well.

#### DISCUSSION.

This series of cases of poliomyelitis in one family, with the circumstances surrounding their origin, forms an instructive illustration of the mode of infection of the disease as brought out by the clinical and experimental study.

In the first place, one child only—the eldest boy, Carey—was exposed in a locality in which poliomyelitis was epidemic. The exposure took place on June 2. Immediately afterwards he returned home, to a village in which no previous case of the disease had occurred, and mingled freely with his younger brothers and sister. The contacts may be considered to have been intimate in that the three male children slept in the same room, two of them in the same bed.

The incubation period in Carey's case was 9 or 10 days, as he was taken ill on June 12. His brother Everett, 6 years younger, developed symptoms 1 day later and passed through what was probably a non-paralytic attack of poliomyelitis. He may be considered as having been infected by Carey some time during the incubation period, and to have exhibited a shorter incubation than his brother. The youngest brother, Dwight, was also freely exposed to both older brothers and exhibited symptoms passing into those indicative of

poliomyelitis 5 or 6 days later than his brothers. Finally, Hazel, the sister, in age between the two older brothers and possibly less freely exposed, developed symptoms and muscular weakness last of all and about 10 days after the eldest brother. The incubation periods of the cases, therefore, probably were 10 days or less, and the order of the attacks was such as to indicate successive infection and not a common one.

The second feature worthy of emphasis is the detection in this one family of two carriers of the poliomyelitic virus by the inoculation test. One (Everett) was discovered to be a carrier probably following a non-paralytic attack. In the instance of Hazel there is no doubt, first that she was discovered to be a healthy carrier, and second that she developed typical poliomyelitis during the period of carriage. Incidentally the nasopharyngeal secretions of Hazel and Everett failed to neutralize the poliomyelitic virus.

If the view that the mode of infection in epidemic poliomyelitis is by way of the nasopharyngeal mucous membrane and is brought about or greatly facilitated through the operation of healthy carriers of the virus, we may well consider whether in the final analysis every case of the disease does not develop from a carrier. At first this may seem startling, and yet it merely means that after contamination of the nasopharynx with the virus, an intervening period exists during which persistence, multiplication, and invasion of the virus take place. In not all contaminated persons does this process become complete; in some the virus may merely persist for a time, in others it may multiply in the nasopharynx (these constitute the healthy carriers of greater or less endurance), while in the exceptional few invasion also occurs. In the latter, symptoms arise, and such individuals compose the class of poliomyelitis cases.

#### SUMMARY.

A family group containing four children of whom all showed in varying degree symptoms of poliomyelitis is described. The source of infection and periods of incubation have been followed. Two of the children were proven by inoculation tests to carry the virus of poliomyelitis in the nasopharynx. Of these, one was detected to be

a carrier after recovering from a non-paralytic attack of the disease, and the other was discovered to be a carrier about 5 days before the initial symptoms, attended later by paralysis, appeared. The original case from which the three others took origin was fatal; the youngest child, after quite a severe onset, was treated with immune serum, and made a prompt and almost perfect recovery. The nasopharyngeal secretions of two of the cases, taken 1 month after the attack, proved incapable of neutralizing an active poliomyelitic virus.

The proposition is presented that every case of poliomyelitis develops from a carrier of the microbic cause, or virus, of poliomyelitis.

#### EXPLANATION OF PLATES.

##### PLATE 55.

FIG. 1. Spinal cord of Monkey B, showing perivascular infiltration and neurophagocytosis.  $\times 90$ .

FIG. 2. Medulla of Monkey B, showing diffuse mononuclear infiltration, nerve cell degeneration, and neurophagocytosis.  $\times 230$ .

##### PLATE 56.

FIG. 3. Intervertebral ganglion of Monkey B, showing infiltrative changes and nerve cell invasion.  $\times 120$ .

FIG. 4. Intervertebral ganglion of Monkey B, showing mononuclear infiltration, nerve cell degeneration, and neurophagocytosis.  $\times 240$ .



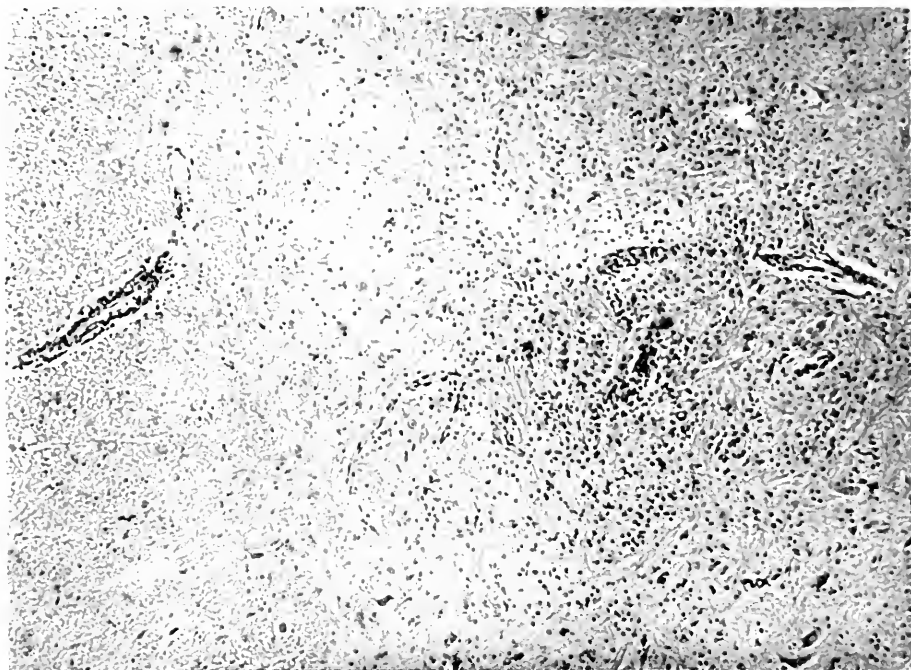


FIG. 1.

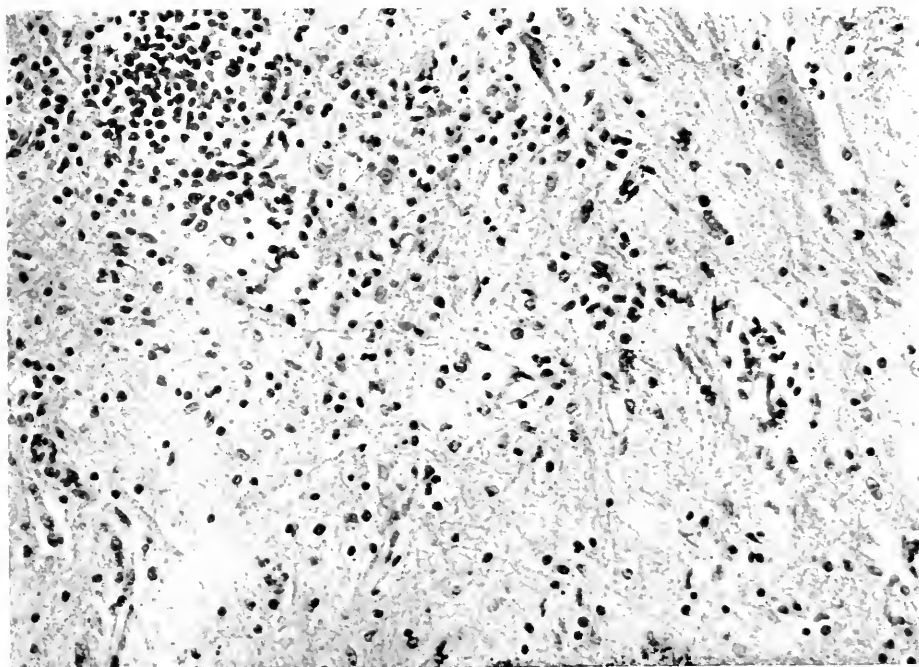


FIG. 2.

(Taylor and Amoss: Carriage of the virus of poliomyelitis.)





FIG. 3.

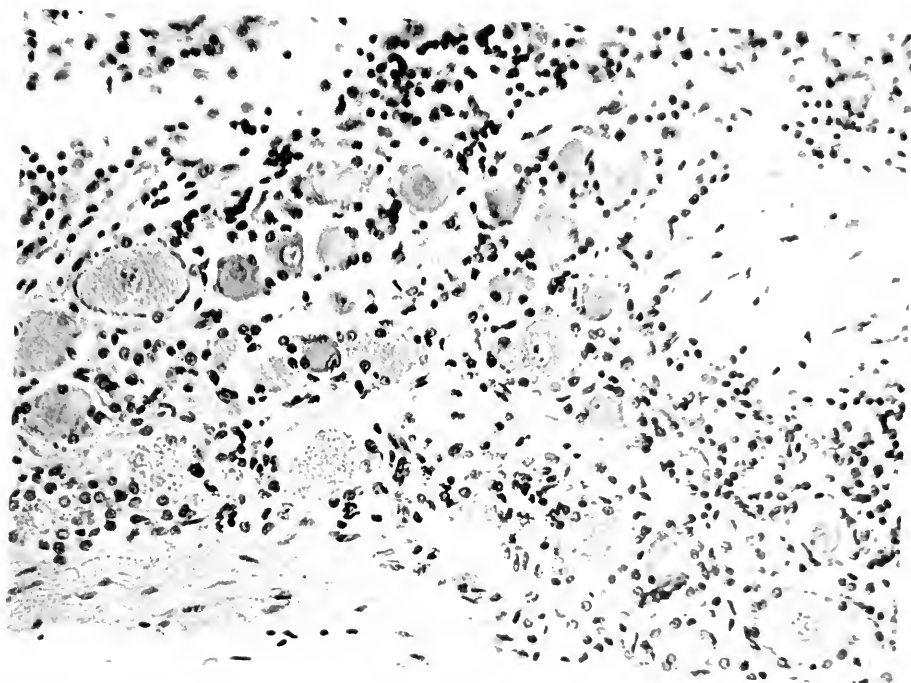


FIG. 4.

(Taylor and Amoss; Carriage of the virus of poliomyelitis.)



75 15

# A NOTE ON PETROFF'S CULTURAL METHOD FOR THE ISOLATION OF TUBERCLE BACILLI FROM SPUTUM AND ITS APPLICATION TO THE EXAMINATION OF MILK.

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## *Examination of Sputum.*

Previous to 1915 the chief means of isolating the tubercle bacillus from sputum was animal inoculation with subsequent cultivation of the recovered organism on some suitable medium, as, for example, blood serum and glycerol egg. Because of the time required, the uncertainty of animal inoculation, and the possibility of modifying the organism by animal passage, this method has proved impractical and unreliable.

Uhlenhuth and Xylander (1908) found that antiformin in certain concentrations is bactericidal for almost all bacteria but the bacillus of tuberculosis, and formulated their well known method for isolating tubercle bacilli in pure culture. Other investigators have adopted their suggestions in whole or in part, but with results as a rule far from satisfactory. Petroff's method was, therefore, indeed welcome. Since the appearance of Petroff's articles (1915) Williams and Burdick (1916) have described a similar method in which they employ a medium of their own. No reports on any duplication of their work have been seen by the writer, but in the few attempts that were made in this laboratory to repeat the work of Williams and Burdick no satisfactory conclusions could be arrived at.

The Petroff medium is prepared easily. It consists of whole egg 2 parts, fresh meat juice (beef or veal) 1 part, glycerol 5 per cent, and gentian violet 1:10,000. It is heated in the inspissator at 85°C. until coagulated, and for an hour at 75°C. on the 2 succeeding days.

The sputum to be examined is digested with an equal amount of 3 per cent sodium hydroxide at 37°C. from 20 to 30 minutes. The

mixture is then neutralized with normal hydrochloric acid and centrifuged. The sediment thus obtained is spread over the surface of the above medium.

In the 1st year that this method was employed Petroff isolated the tubercle bacillus 129 times from 135 specimens of sputum, or in 95.55 per cent of the cases, growth being obtained in 7 to 14 days after inoculation. In several instances he recovered the organism from sputum which had failed to reveal it by staining and direct microscopic examination. He examined feces also, but found it more difficult to isolate tubercle bacilli from this source, being successful only 19 times in the testing of 35 specimens (54 per cent). Although Keilty (1915, 1916) was not so successful as Petroff he expressed the belief that the method would prove itself to be of practical value. He obtained growth from 12 out of 18 samples of known tubercular sputa, only 4 being isolated in pure culture, however. Mitchell and Simmons (1915) examined 35 specimens from which tubercle bacilli were obtained pure in 80 per cent of the cases.

The present investigation is in part a duplication of the work of others on sputum examination. The method was the same as that described by Petroff, except for a few minor details. Some difficulties were at first encountered, such as contamination and drying of the medium, but these were soon largely overcome. All the specimens used were sputa which were procured through the Yale Medical School, and which were pronounced positive as the result of microscopic examination.

Thirty-seven specimens were examined, and from twenty-four, or 64.8 per cent, tubercle bacilli were isolated. The shortest time required for visible growth was 2 weeks, while the average period was from 3 to 4 weeks. In some instances colonies could not be detected in less than 6 weeks after the inoculation. It is apparent from these statements that much more time was required here to obtain the organism in culture than in the work of Petroff. These results, however, coupled with those of other experimenters, appeared so promising that it was deemed desirable to apply the method to the examination of milk, and perhaps later to milk products, meats, sewage, dust, and other agents or media which may be suspected of being infected with tubercle bacilli.

*Examination of Milk.*

With our growing belief in the intercommunicability of bovine and human tuberculosis, the problem of determining the presence of tubercle bacilli in milk by a quick and reliable method is one of great importance. The need of such a method is made all the more apparent by the work of Hess (1908) in New York, Anderson (1909) in Washington, and other investigators in widely separate localities, which showed that at least 6 to 10 per cent of the unpasteurized market milk in this country contains the organism of tuberculosis.

This is not only a public health problem, but also one of great significance from the broader economic standpoint. Tuberculosis often spreads very rapidly in a herd of cattle which then becomes a constant menace to other live stock receiving the infected milk, as well as to young children. According to statistics of the Bureau of Animal Industry about 10 per cent of the dairy cattle in this country are reactors to the tuberculin test, and 9 per cent of all the hogs that were slaughtered under Government inspection in 1916 were found to present evidence of tuberculosis (Mohler and Washburn, 1917). It has been estimated that the aggregate loss to the United States from tuberculosis among farm animals is \$14,000,000 annually.

The only method of determining the presence of tubercle bacilli in milk has been that of animal inoculation. This has proved too expensive, slow, and unreliable, as there is always the possibility of losing the animal from acute non-tubercular infection. Staining and direct microscopic examination of milk or its sediment for tubercle bacilli can receive no serious consideration, on account of the relatively small number of bacilli present.

In the first endeavors to apply Petroff's method to the examination of milk, the milk was artificially infected with a stock culture of *Bacillus tuberculosis*. A small clump of the bacilli was finely mashed in 6 cc. of bouillon or salt solution; after prolonged shaking 4 cc. of this suspension were added to about 85 cc. of cream, from which 4 cc. quantities were then removed and placed in sterile centrifuge tubes. This material was digested with an equal volume of 3 per cent sodium hydroxide solution for 20 to 30 minutes at 37°C. After neutralizing and centrifuging, inoculations from both the fat and

sediment layers in the centrifuge tubes were made on the gentian-violet-egg-meat-juice medium of Petroff.

The first experiments were so successful that larger quantities of milk were inoculated with fewer organisms, and allowed to stand for 12 to 24 hours, some in the ice box and others in the incubator, before the samples were taken for testing. With these less abundantly inoculated samples positive results were obtained less frequently than in the preceding work.

Thirteen samples of milk were artificially infected with tubercle bacilli, four with the human and nine with the bovine type. All of the human and five of the bovine type were recovered, making a total of nine, or 69.2 per cent, positive isolations, a higher figure than that obtained for sputum. From each of these specimens either three or four portions of the milk were taken, making 51 tests in all, from which tubercle bacilli were recovered twenty times, or in 39.2 per cent of the portions tested.

It was impossible to obtain milk from cows having tubercular udders. Samples from only eight tuberculin-reacting animals were available; four of these had reacted 3 years previously, one being negative on second test. The cultural tests with these four samples gave negative results. The other four were from cows that had recently reacted; these likewise proved negative by the cultural method. Another sample of milk which was reported as coming from a cow having generalized tuberculosis was tested, but here again no positive results were obtained, either by the method of Petroff or by guinea pig inoculation.

Through the kindness of the bacteriologists of the New Haven Health Department, 59 samples of milk obtained from widely different sources and taken to the City Laboratory for routine testing were placed at the writer's disposal. Of this number five, or 8.4 per cent, yielded positive growths of tubercle bacilli. As a rule, when four samples were tested one was selected at random for guinea pig inoculation also. In this control experiment 2 cc. of the milk were injected, 1 cc. subcutaneously and the other intraperitoneally. Most of the animals died of acute non-tubercular infection, and of those which lived for several weeks only one showed signs of tubercular infection.



Twenty-nine samples of milk from the State Bacteriological Laboratory have been examined also, all giving negative results. If these samples are included with those obtained from the City Laboratory the percentage of positive cultures is lowered from 8.4 to 5.6.

The above results are fairly comparable with those obtained several years ago in this laboratory, when a bacteriological study of the market milk of New Haven was made, and 6 to 8 per cent of the milk was found to be infected with tubercle bacilli, according to the guinea pig inoculation method. Only six samples of milk purchased in the open market have been examined in the present investigation. The results were all negative.

The colonies of tubercle bacilli which appeared on the medium in the successful isolations from milk were usually small and few in number, hence they were often difficult to detect. However, the bacilli were always found in great numbers in these colonies, and presented the characteristic appearance when stained by the Ziehl-Neelsen method. In one of the successful isolations a guinea pig was inoculated with the acid-fast bacilli. It died 2 weeks after the inoculation, with a loss in weight of 28 per cent and presenting tubercular lesions of the mesentery and liver. The rapidity of action in the test animal confirmed the microscopic examination which showed the bacilli to be of bovine origin.

The morphological characteristics of all the organisms isolated from milk were such as to indicate that they were of the bovine type. The possibility of modifying the medium which has been so successfully used in the isolation of the human type to meet further the requirements of the tubercle bacilli of bovine origin was considered, therefore. In the work of Armand-Delille and his coworkers (1913) luxuriant growths of *Bacillus tuberculosis* were obtained in synthetic media containing certain amino-acids and diamines. It was thought desirable in this investigation to add various amino-acids, and also small amounts of sugar and phosphates to the gentian-violet-egg-meat-juice medium of Petroff. This study has not progressed very far, however, and various combinations of glycocoll, asparagin, glucose, and phosphates are still being tried out. Thus far it appears as if either glycocoll or asparagin favors the growth of the tubercle bacilli of the bovine type.

Certain obstacles must be recognized in attempts to apply the Petroff method to the detection and isolation of tubercle bacilli present in cow's milk, that are not encountered in the examination of sputum. In the first place, the experiments cannot be controlled satisfactorily, as there is as yet no reliable method of identification with which the new method may be compared. Furthermore, the relatively small numbers of tubercle bacilli which are probably present in samples of infected milk render their detection correspondingly difficult. And finally, the slowness and difficulty with which the bovine type of *Bacillus tuberculosis* is made to grow on artificial media are factors which must be reckoned with. However, further investigation of this problem should greatly reduce these obstacles.

#### SUMMARY AND CONCLUSIONS.

1. Tubercle bacilli were isolated by the Petroff method from 64.8 per cent of the sputa which were found to be positive by the direct microscopic method.

2. The organism was recovered from 69.2 per cent of the samples of milk that were artificially infected.

3. Of the milk obtained through the New Haven Health Department Laboratory 8.4 per cent of the samples were found to contain tubercle bacilli, while none of the twenty-nine samples from the State Laboratory gave positive cultures.

4. All the guinea pigs (thirteen), except one, which were used as control animals died without any visible signs of tuberculosis.

5. Petroff's method for the isolation of tubercle bacilli from sputum may be applied successfully to the examination of milk. With slight modifications, the method should prove constant and reliable. The need of such a method for the examination of milk and milk products is apparent.

I wish to express my indebtedness to the members of the City and State Bacteriological Laboratories for supplying samples of milk, to Dr. S. A. Petroff for his suggestions on technique, and finally to Dr. L. F. Rettger for his advice and criticism throughout the course of this investigation.

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# THE BIOLOGICAL IDENTITY OF THE FRIEDLÄNDER BACILLUS.

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Although immunological reactions have been applied to the identification of members of the *Bacillus mucosus capsulatus* group, the refractoriness of these organisms to agglutination by immune sera has stood in the way of a satisfactory classification on this basis.

In spite of the success of Landsteiner,<sup>1</sup> Babes,<sup>2</sup> Klemperer and Scheier,<sup>3</sup> and Bertarelli<sup>4</sup> in agglutinating Friedländer or rhinoscleroma bacilli with immune sera, other investigators (Clairmont,<sup>5</sup> Sicard,<sup>6</sup> Defalle,<sup>7</sup> and Porges<sup>8</sup>) have found these organisms inagglutinable in their native condition, so that recent workers have employed the method of Porges,<sup>8</sup> which is directed towards dissolving the mucoid capsule or hydrolyzing the protein material which holds the bacilli in an inagglutinable suspension. But this treatment by heating in acid solution has been found by Streit<sup>9</sup> to lead to an increase in the susceptibility of the bacterial

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<sup>1</sup> Landsteiner, K., Ueber die Folgen der Einverleibung sterilisirter Bakterien culturen, *Wien. klin. Woch.*, 1897, x, 443.

<sup>2</sup> Babes, V., Ueber das Rhinosklerom, in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 1st edition, 1903, iii, 420.

<sup>3</sup> Klemperer, F., and Scheier, M., Ueber Identität der Ozaena- und der Rhinosklerombacillen mit Friedländer'schen Bacillen, *Z. klin. Med.*, 1902, xlv, 133.

<sup>4</sup> Bertarelli, E., Die Kapselbacillen, insbesondere ihre Systematik und die durch sie bedingten immunitären Reaktionen, *Centr. Bakteriolog., 1te Abt., Ref.*, 1906, xxxvii, 343.

<sup>5</sup> Clairmont, P., Differentialdiagnostische Untersuchungen über Kapselbakterien, *Z. Hyg. u. Infektionskrankh.*, 1902, xxxix, 1.

<sup>6</sup> Sicard, A., *Compt. rend. Soc. biol.*, 1899, i, series 11, 813.

<sup>7</sup> Defalle, W., Sur le rôle de l'enveloppe des microbes, *Ann. Inst. Pasteur*, 1902, xvi, 602.

<sup>8</sup> Porges, O., Ueber die Agglutinabilität der Kapselbakterien, *Wien. klin. Woch.*, 1905, xviii, 691.

<sup>9</sup> Streit, H., Zur Frage der Agglutinierbarkeit von Kapselbacillen, *Centr. Bakte iol., 1te Abt., Orig.*, 1906, xl, 709.

suspension to normal sera and to spontaneous agglutination, as well as to immune sera. For this reason as well as for the variation in the length of treatment required to make different strains agglutinable, the method has not, in the hands of Streit<sup>9</sup> and Beham,<sup>10</sup> been found suitable for diagnostic purposes.

Cultures made to grow in a dry or capsule-free state, by development at low temperature (110°C.), or on potato agar, or by an artificial selection of the smaller and dryer colonies for several generations, have been found by Streit and Beham respectively to be agglutinated by immune sera. Since these dry cultures have been found by these writers, and by von Eisler and Porges,<sup>11</sup> to regain their capsulated mucoid state on animal passage, this simpler method does not appear suited to the recognition of bacilli in their most characteristic condition, as direct from the animal body.

#### EXPERIMENTAL.

The present study was undertaken as part of an immunological study of the capsulated Gram-negative bacilli. The material consisted of fifty strains, some isolated in this laboratory, and the rest collected from various sources.<sup>12</sup> All the organisms could be placed in the group comprising Friedländer's bacillus, *Bacillus mucosus capsulatus*, etc., and *Bacillus acidi lactici*, *Bacillus aerogenes*, etc.

For comparison of the cultural characteristics the organisms were grown in parallel streaks on dextrose agar in large (15 cm.) plates. In this way it has been possible to distinguish a Friedländer type, with colonies gray, translucent, of fluid, often syrupy consistency, and transparent watery margins, from an *aerogenes* type showing variations but having in common opaque ivory-white colonies, of a more pasty consistency. This cultural distinction is the main difference between the two organisms described by Kruse,<sup>13</sup> and may be

<sup>10</sup> Beham, L. M., Die agglutinatorischen Eigenschaften der Kapselbacillen und Anwendung der Serumagglutination bei den Trägern von Kapselbacillen, *Centr. Bakteriolog., 1te Abt., Orig.*, 1912, lxvi, 110.

<sup>11</sup> von Eisler, M., and Porges, O., Ueber die Differenzierung der Kapselbakterien mit Hilfe agglutinierender und präcipitirender Immunsere, *Centr. Bakteriolog., 1te Abt., Orig.*, 1906, xlii, 660.

<sup>12</sup> For these I am indebted to Dr. W. Rothberg of the American Museum of Natural History, Dr. J. G. Dwyer of the College of Physicians and Surgeons, Dr. E. G. Stillman of the Hospital of The Rockefeller Institute for Medical Research, and Miss M. Olmstead of the Presbyterian Hospital.

<sup>13</sup> Kruse, W., in Flüggé, C., Die Microorganismen, Leipsic, 3rd edition, 1896, pt. 2, 185.

regarded as the basis of the classifications of Clairmont<sup>5</sup> and of Strong.<sup>14</sup>

Eleven of our fifty strains belong to the Friedländer type. The cultural difference in our experience has been constant both for young and old cultures, but can be recognized surely only when the different cultures are grown upon the same plate. The fluidity of the Friedländer strains varies somewhat upon different samples of media, and on blood agar the growth is more luxuriant and opaque, but the mucoid characteristic is retained in all cultures and we have not been able to observe distinct moist and dry growing phases. Two further strains show a translucent mucoid growth on dextrose agar, but resemble *Bacillus coli* on plain agar. One of these, No. 12, from the intestine forms gas from lactose; the other, No. 48, from a normal mouth forms acid but no gas on lactose and saccharose, and can be distinguished by this means also from the eleven type strains. It is possible that such variable strains as these have been observed by authors who note a drying out of the cultures on long continued cultivation or a relation between mucoid growth and the water and sugar content of the media (Fürst<sup>15</sup>). Other organisms of a more colon-like growth are distinguished culturally without difficulty.

The source of the Friedländer type strains, and their fermentation reactions with dextrose, lactose, and saccharose are given in Table I.

Rabbits have been immunized with killed suspensions of agar growths of Strains 3, 24, and 27. Intravenous inoculations were made every 4 or 5 days. Eleven of the fourteen animals died in the course of the treatment, all but two dying in from 2 to 6 hours after a dose slightly larger than the previous one. At autopsy the findings of distended lungs, petechial hemorrhages of the serous surfaces, and greatly lengthened coagulation time of the blood have pointed to an anaphylactic death.

In the blood of the seven animals examined it has been possible to demonstrate agglutinins for the homologous strain after 19 to 23 days of immunization. For testing agglutination 0.1 cc. of a broth

<sup>14</sup> Strong, L. W., Ueber die Kapselbacillen, *Centr. Bakteriöl., 1te Abt.*, 1899, xxv, 49.

<sup>15</sup> Fürst, T., Untersuchungen über Kapsel- und Hüllenbildungen bei den sogenannten Kapselbakterien *Centr. Bakteriöl., 1te Abt., Orig.*, 1910, lvi, 97.

TABLE I.

Series No.	Source.	Dextrose.	Lactose.	Saccharose.
3	Bronchopneumonia.....	Acid and gas.	No acid or gas.	Acid and gas.
4	Lung.....	" " "	" " " "	" " "
17	Ropy milk.....	" " "	" " " "	" " "
23	Ozena.....	" " "	" " " "	" " "
24	Normal mouth.....	" " "	" " " "	" " "
25	Lobar pneumonia, blood.....	" " "	" " " "	" " "
27	Meningitis.....	" " "	" " " "	" " "
36	Sputum; child with pneumonia.....	" " "	" " " "	" " "
37	Sputum; child with pneumonia.....	" " "	" " " "	" " "
47	Normal mouth.....	" " "	" " " "	" " "
50	" " .....	" " "	" " " "	" " "

culture has been used, in a total volume of 0.5 cc. of serum dilution. The titer has been low for most sera not exceeding 1:10, but for two of the three animals that survived a longer immunization values of 1:40 to 1:60 were found. The addition of the bacteria to concentrated serum, 1:1 or 1:5 dilution, produces an almost immediate coarse flocculation which soon settles and is compacted into a firm disc in the bottom of the tube. In higher dilutions the flocculation is finer, slower in appearing, does not form so firm a disc, and in the highest dilutions does not produce a clearing of the tube.

This result presents a striking similarity to the agglutination of *Pneumococcus mucosus* by the immune serum recently described by Wadsworth and Kirkbride.<sup>16, 17</sup>

Quantitative relations between serum and culture are strictly maintained so that 0.1 cc. of serum will cause complete agglutination of the same amount of culture whether it is in a volume of 0.2 or of 1.0 cc. The concentration affects only the speed of the reaction. The stability of Friedländer bacillus emulsions has been noted by Porges, Streit, and Bertarelli, and is so marked that clearing of broth or saline emulsions is impossible even with the highest centrifuge

<sup>16</sup> Wadsworth, A. B., and Kirkbride, M. B., A note on the production of anti-pneumococcus sera, *J. Exp. Med.*, 1917, xxv, 629.

<sup>17</sup> This immune serum was kindly placed at my disposal by the authors.



speed obtainable. This is due, as Porges<sup>18</sup> explains, to the presence of dissolved bacterial protein substance. The soluble material is present in broth cultures, which are found considerably less agglutinable than equally turbid suspensions of bacteria washed in a Berkefeld filter or by centrifugation. The following protocol illustrates this point:

Serum 24-A	1 : 10	1 : 20	1 : 40	: 60
Culture 24 (24 hr. broth culture).....	±	—	—	—
Emulsion of washed bacteria from same culture.....	+++	+	±	—

The dissolved protein may be obtained by filtration from broth cultures as soon as turbidity has begun to appear, as early as 4 hours after planting. This substance reacts specifically with immune serum to give a heavy flocculent precipitate so that it is probable that it hinders the outflocking of the bacteria mainly by altering the quantitative relations between serum and substrate.

The viscid fluid obtained by washing out the peritoneal cavity of an infected animal is readily flocculated by immune serum, and capsules can be demonstrated in the agglutinated mass from this, as well as from broth cultures and washed bacterial emulsions. Examined in the hanging drop, the capsules appear in saline solution or normal serum only as halos of refraction, but on the addition of concentrated immune serum come immediately into prominence, swell to half the diameter of a red blood cell, and show a sharp non-refractile periphery. A similar observation was made by Landsteiner,<sup>1</sup> but appears to have been overlooked by later investigators. In weaker sera, agglutination may take place without the occurrence of this change. By the fuchsin-copper sulfate method the swollen capsules take a uniform heavy stain.

These observations do not support a morphological conception of the capsule as an impermeable envelope; they indicate rather that the

<sup>18</sup> Porges, O., Ueber die Beziehungen zwischen Bakterienagglutination und Ausflockungserscheinungen der Kolloide, *Centr. Bakteriол., Ite Abt., Orig.*, 1906, xl, 133.

peculiar properties of the Friedländer bacillus protein determine the nature and the extent of its reaction with immune serum.

The eleven strains belonging to the Friedländer cultural type were agglutinated by immune sera developed against strains from pneumonia, from meningitis, and from a normal mouth. For a given serum, the agglutination titer was approximately the same for all strains. There was no evidence on which to base a subgrouping; unfortunately the supply of high titer serum was exhausted before complete absorption experiments could be applied to this point. The remaining thirty-nine strains representing different cultural types were not agglutinated by any immune serum even in 1:1 dilution.

#### CONCLUSION.

We conclude therefore that this series of eleven lactose-negative organisms of the Friedländer type, grouped together by Perkins<sup>19</sup> on the basis of fermentation reactions represents a single biological group. It can be distinguished from *Bacillus aerogenes* and other similar bacilli by cultural, fermentative, and serological reactions. There appears to be a close analogy between this group and *Pneumococcus mucosus* in the possession of a fixed cultural type, and the behavior toward immune serum. Both represent apparently a single biological group. Unfortunately no immune sera have been developed against the two strains that grew in moist and dry phases; it is possible that with immune sera for these light could be thrown on the relation suggested by Fitzgerald<sup>20</sup> that the capsulated bacilli represent a parasitic development of the *Bacillus coli* group.

I wish to express my indebtedness to Professor Zinsser for his suggestions, and my appreciation of the interest and advice of Professor J. Gardner Hopkins, both of the Department of Bacteriology of the College of Physicians and Surgeons of Columbia University.

<sup>19</sup> Perkins, R. G., *J. Infect. Dis.*, 1904, i, 241.

<sup>20</sup> Fitzgerald, J. G., *J. Infect. Dis.*, 1914, xv, 268.

# THE EFFECT OF ALCOHOL ON THE REPRODUCTIVE TISSUES.

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PLATES 57 TO 60.

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That alcohol may produce distinct anatomical changes in the testicles of those who use it in excess has been observed from time to time by pathologists for many years, and yet this fact does not seem to be widely known or generally taken into account in considering the effects of alcoholism.

## HISTORICAL.

As long ago as 1837, Rösch<sup>1</sup> described degenerative changes in the testicles of alcoholics, and Lancereaux described parenchymatous degeneration of the seminal tubules. In 1877 Schlemmer<sup>2</sup> discussed the effects of alcoholism on the testicles, and noted that it often caused fatty degeneration of the seminal epithelium, decrease in the number of spermatozoa, and pigmentation of the seminal fluid. Rheinstaedter<sup>3</sup> (1879) and Curschmann<sup>4</sup> attributed loss or decrease in testicle function to chronic alcoholism, the former stating that in his experience excessive beer drinking had a greater effect than wines or distilled liquors.

Busch<sup>5</sup> (1882) studied the testicles in 100 consecutive autopsies of adult males and found 27 without spermatozoa, 39 with few, and 34 with many. Although phthisis and other chronic diseases furnished many cases of azoospermia, he observed that this condition occurred frequently in alcoholics, especially when associated with cirrhosis.

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<sup>1</sup> Rösch, cited by Forel.<sup>13</sup>

<sup>2</sup> Schlemmer, A., *Vrtljschr. gerichtl. Med.*, 1877, xxvii, 444.

<sup>3</sup> Rheinstaedter, cited by Busch.<sup>5</sup>

<sup>4</sup> Curschmann, cited by Busch.<sup>5</sup>

<sup>5</sup> Busch, A., *Z. Biol.*, 1882, xviii, 496.

In 1898 Simmonds<sup>6</sup> reported the study of 1,000 male bodies, of which 125 showed azoospermia. As previously observed by Busch, Simmonds found chronic diseases responsible for many cases, the incidence of azoospermia in cancer cases being 7 per cent, in chronic nervous diseases 14 per cent, in phthisis 18 per cent, while of the chronic alcoholics 60 per cent had no spermatozoa. Of all autopsied males, 5 per cent were sterile through the use of alcohol, as against 3.3 per cent from genital diseases. He also found that mechanical occlusion of the seminal tract, even of years' duration, seldom causes atrophy of the testicles or absence of spermatozoa in the tubules.

Bertholet<sup>7</sup> (1909) made a report of the findings in 75 bodies of men dying at from 15 to 91 years of age, of whom 39 were habitual drinkers. Of the 39 alcoholics, only 2 had testicles that did not show atrophy of the parenchyma and sclerosis of the stroma. The most marked change occurred in alcoholics who died of tuberculosis or cirrhosis. Of the two alcoholics without atrophy, one was a man but 24 years old, and the other died of an acute intestinal obstruction. Bertholet describes the changes in detail. Generally the testicles are noticeably small and firm, because of atrophy of the epithelium and increase in the fibrous tissue. The spermatogonia are highly atrophic, the nuclei cannot be stained distinctly with hemalum, and it is impossible to distinguish between spermatocytes and spermatids, because they are all in the resting stage and show no mitosis or spermatozoa production. The cells of Leydig, on the other hand, seem to be increased in size, number, and staining intensity of the cytoplasm. The basal membrane is much thickened by concentrically arranged lamellæ and spindle cells; there is also an increase in the connective tissue between the tubules, with sometimes a slight round cell infiltration. The atrophy and azoospermia was complete in 24 of the 37, including 7 who died of cirrhosis; in but 4 was the change of only insignificant degree. Although similar changes of slight degree were found in non-alcoholics, the advanced changes were seen only in those who drank heavily. Even in senility the atrophy and fibrosis were less marked than in the alcoholics.

Since the reports of Simmonds and Bertholet, a few other papers have appeared corroborating their observations in all essentials, although not all investigators on alcoholism have considered the sex cells. Thus Fahr,<sup>8</sup> in his study of the anatomical changes in 309 alcoholics, makes no mention of the generative tract or the occurrence of changes in these tissues in the guinea pigs that he subjected to experimental alcoholism.

Weichselbaum and Kyrle<sup>9</sup> (1912) have furnished one of the best contributions to this subject, having carefully studied the testicles of 67 habitual alcoholics. They entirely support Simmonds' and Bertholet's conclusion that chronic

<sup>6</sup> Simmonds, *Berl. klin. Woch.*, 1898, xxxv, 806.

<sup>7</sup> Bertholet, E., *Centr. allg. Path. u. path. Anat.*, 1909, xx, 1062.

<sup>8</sup> Fahr, *Verhandl. deutsch. path. Ges.*, 1909, xiii, 162.

<sup>9</sup> Weichselbaum, A., and Kyrle, J., *Sitzungsb.-Ber. Akad. Wien.*, 1912, cxxi, 51.

alcoholism constantly causes retrogressive changes in the testicles independent of senility or chronic diseases, even to the point of complete loss of the seminal epithelium. Also they corroborate the observation that the most marked changes occur in the testicles in alcoholism with cirrhosis. They too find that tuberculosis and cancer may produce similar changes, usually of mild degree, but alcoholism of itself produces marked changes without the coexistence of any other disease. Their paper contains a detailed description of the histological changes observed.

Because of the repeated observation that alcoholics with cirrhosis show especially marked changes in the testicles, Schopper<sup>10</sup> investigated the effect of liver injury on the testicles. He found that animals (rabbits, dogs, and rats) from which a large portion of the liver had been removed, or in which the liver had been injured by exposure to Roentgen rays, exhibited marked changes in the testicles. These changes consisted in a cessation of spermatogenesis, transudation into the interstitial tissue, desquamation of the epithelial cells with formation of multinucleated cells, and even complete loss of the epithelium with increase in the interstitial cells.

In connection with his studies on the effect of parental alcoholism on the development of the offspring, Stockard<sup>11</sup> stated that guinea pigs given alcohol for as long as 19 months by inhalation, and therefore in unknown quantity, were still good breeders, and when killed showed no changes in the testicles. Ovaries of females similarly treated also showed no recognizable structural changes. On the other hand, Riddle and Basset<sup>12</sup> found that doves and pigeons made to inhale alcohol 1 to 2 hours daily for a few weeks produced eggs with yolks smaller than those in the eggs laid before the alcoholism. In general, however, the possible anatomical changes in the ovaries seem not to have been considered by pathologists, and the only mention we can find of this topic is contained in the review of Forel,<sup>13</sup> who says, "more recently Bertholet has also demonstrated atrophy of the ovaries and ova of an alcoholic woman," but no reference is given and we have been unable to locate this statement in Bertholet's writings.

The only investigation of the influence of experimental alcoholism on the testicles of animals that we can find, beyond Stockard's negative results, is described by Bouin and Garnier,<sup>14</sup> and unfortunately this report concerns only an isolated observation. It is stated by these authors that in the course of experiments on the effect of chronic alcoholism on the white rat, marked alterations were observed in the testicles of two animals that had been fed alcohol for 8 and 11 months respectively. No mention is made of how many animals were studied in

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<sup>10</sup> Schopper, K. J., *Frankfurter Z. Path.*, 1911, viii, 169.

<sup>11</sup> Stockard, C. R., *Arch. Int. Med.*, 1912, x, 374; *Arch. Entwcklungsmechn. Organ.*, 1912-13, xxxv, 3. Stockard, C. R., and Papanicolaou, G., *Am. Naturalist*, 1916, i, 65.

<sup>12</sup> Riddle, O., and Basset, G. C., *Am. J. Physiol.*, 1916, xli, 425.

<sup>13</sup> Forel, A., *Münch. med. Woch.*, 1911, lviii, 2596.

<sup>14</sup> Bouin, P., and Garnier, C., *Compt. rend. Soc. biol.*, 1900, lii, 23.

all, or whether the other animals showed any changes in the testicles. In these two animals there was marked atrophy of the seminal tubules, often with almost complete disappearance of the germinal epithelium, and in none was the epithelium normal. Between the tubules was found an accumulation of lymph, but no fibrosis or arteriosclerosis. In the lumen of the tubules few spermatozoa were found, but there was much desquamation of the epithelium, and often fusion of the cells into masses. Pyknosis and karyolysis were common, and the Sertoli cells often showed amitotic division. Before degenerating completely the germinal cells pass through various stages of altered vitality, which, the authors suggest, explains the frequency of abnormalities in the offspring of alcoholics. We cannot find that these authors have published elsewhere further details of their investigations in this field.

### *General Physical Changes Due to Alcohol.*

In a series of rats fed alcohol for varying periods of time to determine the effect of alcohol on the psychology of their offspring, an opportunity was given to observe the effect of chronic alcoholism on these animals.<sup>15</sup> Altogether we examined 15 male rats thus treated, 6 male rats of the second generation derived from them, 18 male rats of the third generation, and 8 male rats of the fourth generation. Approximately the same number of females was also studied. From each animal, with a few exceptions, the following organs were studied: lung, liver, kidney, spleen, stomach, heart, and sex glands. The animals were all killed, while in apparent health, with illuminating gas. They were all of about the same age, 6 to 9 months, except in the case of the rats fed 0.5 cc. for 10 months; these rats were 13 months old. All had been fed and cared for under identical conditions except for the administration of alcohol admixed with the food in quantities of from 0.25 cc. to 2.25 cc. per day, for periods varying from 2 to 10 months. The effect of the alcohol on the rats receiving it was, except for those fed for very long periods, to lessen learning capacity in almost direct proportion to the amount of the dose and the duration of the period during which it was administered.

The effect on body weight was also marked. Animals receiving 2.25 cc. *per diem* rate either gained weight far more slowly than the normal animals, or, as in some instances, actually lost weight though in the

<sup>15</sup> The results of the psychological study will be published shortly in *Psychol. Monog. Suppl.*

growing period. As for the offspring, the observed physical effects were, in the case of the offspring of rats receiving 0.5 cc., a decrease in size and lessened fertility. This was accompanied by a marked decrease in learning capacity. The offspring of animals receiving 0.25 cc. showed no marked physical changes and were, as to intelligent behavior, generally on a par with, if not actually superior to, the offspring of normal animals.

The microscopic changes in all structures except the sex glands may be summarized briefly. Nearly all showed more or less pulmonary infection of the type common in rats, consisting in the less advanced stages of a chronic bronchitis with much cellular infiltration and hyperplasia of the bronchial walls. Many showed extension of the process to the adjacent alveoli with a frank mucopurulent bronchopneumonia. The degree of this change seemed to have little effect on the conditions in the other organs, and bore no relation whatever to the changes observed in the sex glands.

In the liver there were often varying degrees of hydropic vacuolization of cells, this condition occurring in about half the specimens. Focal necrosis was found in about one-fourth, severe in but a few. These changes bore no reference to the severity of the lung infection or to whether alcohol had been administered, as shown by tabulation and comparison of the results. Occasionally a slight periportal round cell infiltration was found, but this also was independent of other conditions. Even in the rats that received large quantities of alcohol for some months, the livers showed no more change than in the others. There was nothing found at all resembling cirrhosis.

The kidneys showed commonly a slight degree of vacuolization of the tubular epithelium, which was possibly slightly more general in the alcohol animals than in the others. Two animals in which the kidneys showed slight formation of granular casts had received much alcohol. That the alcohol was the cause of the renal changes is probable, especially in view of the great amount of cast formation commonly observed in the urine of patients with acute alcoholism, but this is by no means certain.

In the stomachs of a few of the rats receiving alcohol there seemed to be slightly more round cells in the mucosa and submucosa than normally, but the changes were not marked enough to be considered as

distinctly inflammatory. In two of the alcoholized rats the gastric glands showed more mucus content than was seen in any of the other animals, and hence this change may be ascribable to the alcohol. The slight effect of the alcohol on the stomach of the rats corresponds to the relatively normal condition of the gastric mucosa of human alcoholics observed by Hirsch.<sup>16</sup>

The heart, spleen, and adrenals usually showed no changes, and in no instance changes that occurred in the alcoholized rats and not in the others.

### *Pathological Changes in the Reproductive Tissues.*

Only the testicles showed definite changes, but here the effects were so marked, and so nearly constant, that they stand out conspicuously as the result of the alcohol feeding. Of the fifteen male rats treated with alcohol, the testicles of not more than two or three failed to show noteworthy changes, and only one could be called normal. Several different types of changes were observed, and one that attracted attention at once was an apparent decrease in the size of the seminiferous tubules, and in several instances a distinct decrease in the size of the testicle itself. Measurements were made of the tubules in the testicles from thirty-seven rats, twenty-five tubules being measured from each testicle to secure adequate representation, and then the average diameter was calculated.<sup>17</sup> The results of measurements in the several groups are given in Table I.

TABLE I.

No. of animals.	Minimum diameter.	Maximum diameter.	Average diameter.
6, 2nd generation rats.....	16.00*	19.16	17.26
18, 3rd " ".....	15.08†	22.60	17.50
8, 4th " ".....	15.56	18.32	17.08
15, alcoholized rats.....	10.00	18.24	14.65

\* The measurements as recorded are the number of ocular micrometer spaces, not translated into microns. Each ocular micrometer space = 0.0155 mm.

† One very abnormal testicle in this series, measuring but 12.12, is not included.

<sup>16</sup> Hirsch, E. F., *Arch. Int. Med.*, 1916, xvii, 354.

<sup>17</sup> For these measurements we are indebted to Miss C. L. Chapin.



These measurements show at once that there is a distinct decrease in the size of the seminal tubules of the rats that had received alcohol, although in a few rats this change is not present. However, as Table II shows, but two of the fifteen rats that received alcohol had as large tubules as the average seminal tubules of their descendants that had not received alcohol. It also shows that the decrease in size does not vary directly with the amount of alcohol the rat had received. The tubules of normal rats of other strains corresponded in size to the tubules of the second, third, and fourth generation rats.

TABLE II.

Rat No.	Daily dose.	Time.	Diameter of tubules.	Histological changes summarized.
	cc.	mos		
1	0.25	2	13.40	Many defective spermatozoa; number decreased.
2	0.25	2	15.04	Spermatozoa decreased.
3	0.25	4	13.68	Few spermatozoa; mostly defective.
4	0.5	3	17.12	Nearly normal.
5	0.5	3	16.16	A few inactive tubules; mostly normal.
6	0.5	3	18.24	" " " " " "
7	0.5	10	11.32	Marked atrophy with intertubular edema; almost no spermatozoa, and these defective.
8	0.5	10	16.76	Much intertubular edema; most of tubules nearly normal, but number of spermatozoa decreased.
9	2.0	6	14.32	Very few spermatozoa; much desquamation.
10	2.0	6	14.16	Much desquamation and degeneration of epithelium; small number of spermatozoa.
11	2.25	3	10.00	No spermatozoa; great desquamation and degeneration of epithelium.
12	2.25	3	13.56	Only one-third to one-half the tubules contain spermatozoa, and few of these are normal.
13	2.25	6	15.84	Some edema; spermatogenesis much reduced.
14	2.25	6	13.88	Much atrophy and edema; spermatogenesis about 50 per cent of normal.
15	2.25	6	16.24	About three-fourths of the tubules nearly normal.

From Table II it will be seen that the least changes were found in the three rats (Nos. 4, 5, and 6) that received 0.5 cc. of alcohol for 3 months, while much more marked changes occurred in the three animals that received but 0.25 cc. of alcohol. Also, three of the rats that received the relatively enormous dosage of 2.25 cc. per day for

6 months, which would be equivalent to a man's taking over 2 quarts of whisky daily for a large part of his life, showed less change in the testicles than some of the rats that received somewhat less alcohol.

The changes produced by alcohol seem to take place in definite order. At first the spermatocytes seem to be normal in number and appearance, but there is soon an increase in the number of spermatids in the tubules with a decrease in the number of spermatozoa (see Fig. 1, showing a normal testicle, and Figs. 2 and 3, showing the testicles of rats which received 2 cc. and 2.5 cc. respectively). At the same time, or earlier, there is observed a greater diminution in the number of complete spermatozoa with tails than in the number of sperm heads. Not all the tubules in the testicles show the same degree of change, except in the most advanced cases (Fig. 4). In the more moderately affected testicles one finds some tubules nearly or quite normal, while others show merely a few active spermatocytes with the lumen packed with spermatids without spermatozoa or even spermatozoa heads (Fig. 5). Apparently the first effect of the alcohol is to render the formation of spermatozoa incomplete, so that the heads are formed without normal tails. In accordance with this, in one of these testicles we usually find the number of spermatozoa in the tubules of the epididymis much less than normal, and less in proportion than the number of spermatozoa seen in the seminiferous tubules, suggesting that the latter are too imperfect to migrate to the epididymis. The next effect of the alcohol seems to be to prevent the transformation of the spermatids into spermatozoa, whereby the tubules become filled with accumulated spermatids, with but a few spermatozoa or none at all. These spermatids then undergo a process of degeneration, so that they lose their nuclear stain and become granular. In the most advanced stages the tubules contain but the marginal cells, with few or no spermatocytes or spermatids (Fig. 6). Occasionally in the lumen of tubules showing advanced degeneration are found large cells with many nuclei or chromatin masses.

The degree of atrophy does not exactly parallel the decrease in functional activity, but usually the diameter of the tubules corresponds somewhat with the loss of function. When the tubules are atrophied there is commonly some compensatory accumulation of

serous fluid in the interlobular spaces, an edema *ex vacuo* (Fig. 4). In no instance have inflammatory changes been seen, and no marked fibrosis, although in some instances there has been an apparent slight increase in the thickness of the basement membrane of the tubules. We have noted no definite multiplication or other changes in the interstitial cells of Leydig, although when the tubules shrink apart these cells become more evident. No arterial changes were observed.

The epididymis generally reflects, in the number of spermatozoa it contains, the degree of functional impairment of the testicle (Figs. 7 and 8). In two of the rats there was also noteworthy desquamation of the epithelial lining of the ducts. Other changes were not observed in the epididymis.

Although the ovaries of the alcoholized females, and of the offspring of alcoholized parents, were examined in considerable numbers, it is not possible to make any positive statement as to the effect of alcohol on this organ. The changes of ovulation, pregnancy, and corpus luteum formation produce such great structural alterations in the ovary that we were unable to determine satisfactorily what changes were caused by the alcohol. There were two distinctly atrophic ovaries found in females that had had alcohol, and in several alcoholic females the ovaries were poor in ova in the sections examined.

Without study of serial sections of every ovary no conclusions of value could be reached from the material, and we were unable to undertake this task.

#### SUMMARY.

Administration of alcohol in the food of male white rats for 2 or more months, in daily quantities of 0.25 to 2.25 cc., results almost constantly in the appearance of marked degenerative alterations in the testicles. These changes affect the steps of spermatogenesis in inverse order to their occurrence, so that for some time before sterility and complete aspermia result, the animal is producing spermatozoa with all possible degrees of abnormality and deficiency. The possible relation of this abnormal spermatogenesis to the production of defective offspring is obvious. Individual rats show marked differences in the degree of change produced by equal amounts of alcohol. The

fibrous, interstitial, and vascular elements of the testicle are not affected, except for intertubular edema compensating for tubular atrophy. These experimental observations harmonize with the necropsy findings in human alcoholics. No other tissue was found to be noticeably affected by the alcohol; especially to be remarked is the absence of cirrhosis or fatty infiltration in the liver.

#### EXPLANATION OF PLATES.

##### PLATE 57.

FIG. 1. Normal testicle of a rat.  $\times 58$ .

FIG. 2. Testicle of a rat which had received 2 cc. of alcohol daily for 6 months. Marked loss of germinative epithelium and absence of spermatogenesis are shown, without much decrease in size. Many tubules in this testicle also show spermatogenesis.  $\times 58$ .

##### PLATE 58.

FIG. 3. Testicle of a rat which had received 2.25 cc. of alcohol daily for 3 months. Marked atrophy of the cellular content, decrease in size of the tubules as a whole, and complete absence of spermatogenesis are shown.  $\times 58$ .

See higher power of the same in Fig. 6.

FIG. 4. Testicle of a rat which had received 0.5 cc. of alcohol daily for 10 months. Marked decrease in size of the tubules with intertubular edema.  $\times 58$ .

##### PLATE 59.

FIG. 5. Tubule of a rat which had received 0.25 cc. of alcohol daily, showing absence of spermatozoa and accumulation of spermatids. This is one of the early stages of the degeneration of the testicular tubules.  $\times 309$ .

FIG. 6. Higher power of Fig. 3. Later stage of degeneration. The tubules contain no spermatozoa or spermatids, and almost no spermatocytes.  $\times 309$ .

##### PLATE 60.

FIG. 7. Normal epididymis of a rat. Tubules filled with spermatozoa.  $\times 58$ .

FIG. 8. Epididymis of a rat which had received 2 cc. of alcohol for 6 months, showing great atrophy and no normal spermatozoa.  $\times 58$ .

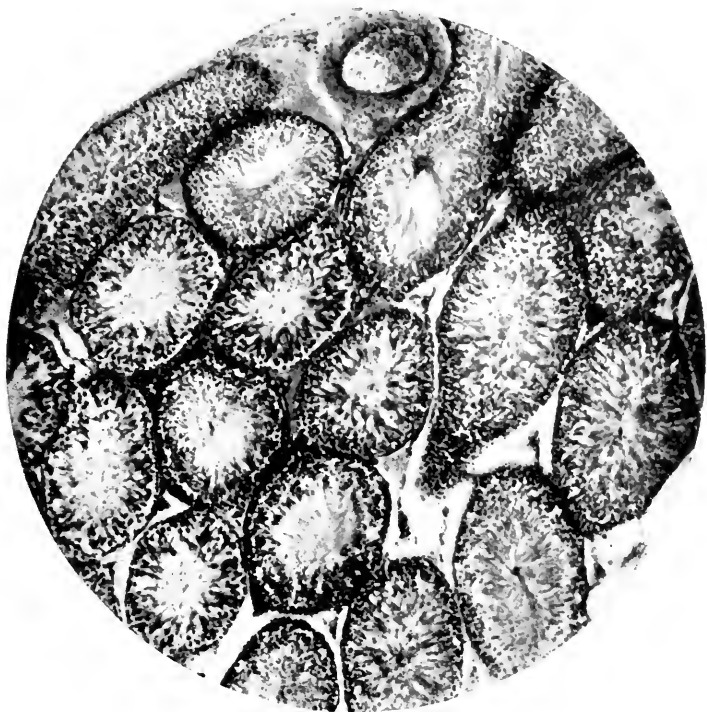


FIG. 1.

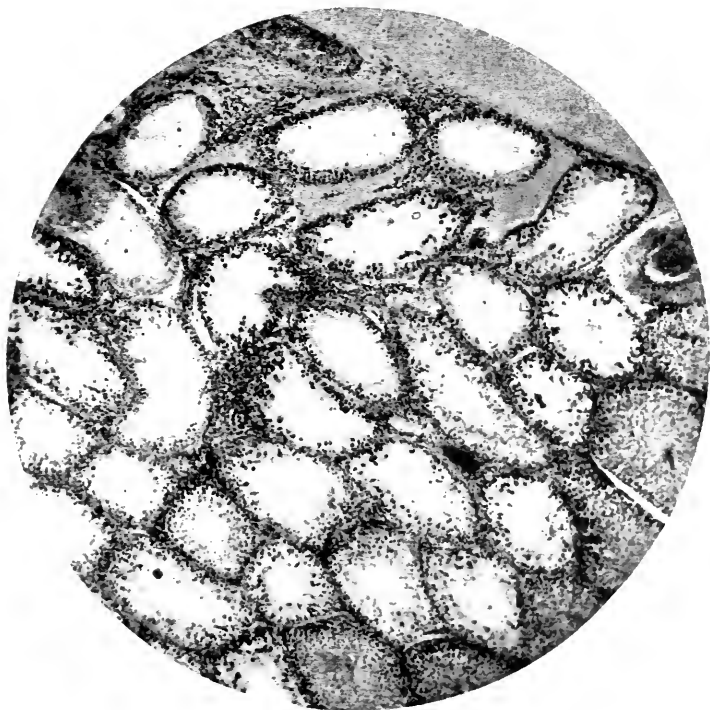


FIG. 2.

(Arlitt and Wells: Effect of alcohol on reproductive tissues.)



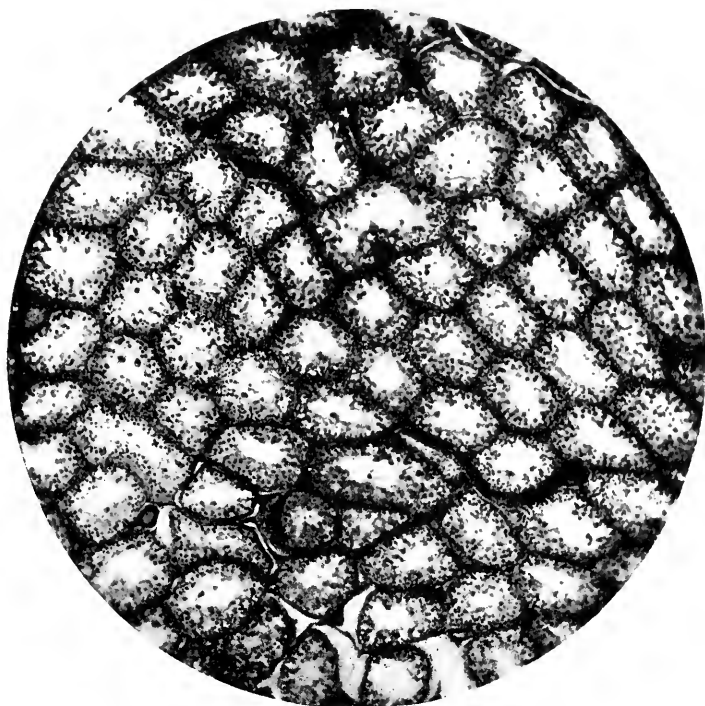


FIG. 3.

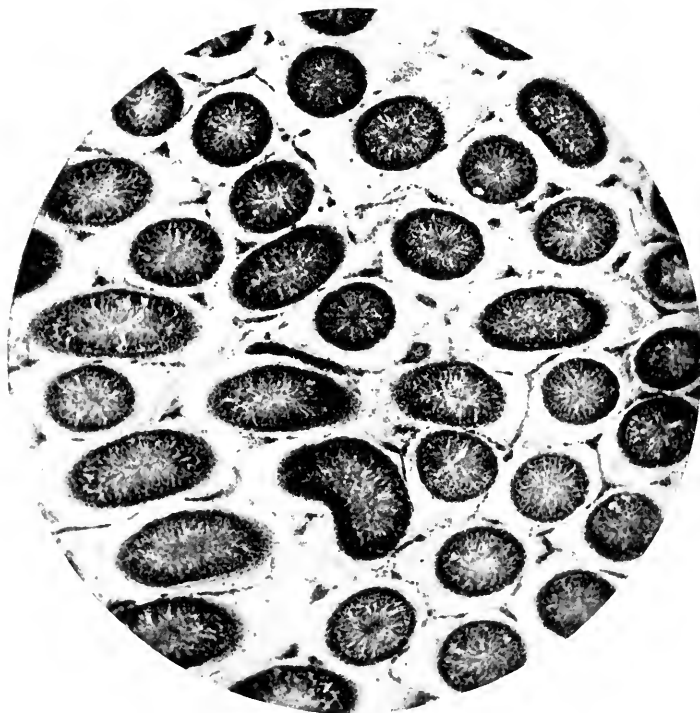


FIG. 4.

(Arlitt and Wells: Effect of alcohol on reproductive tissues.)





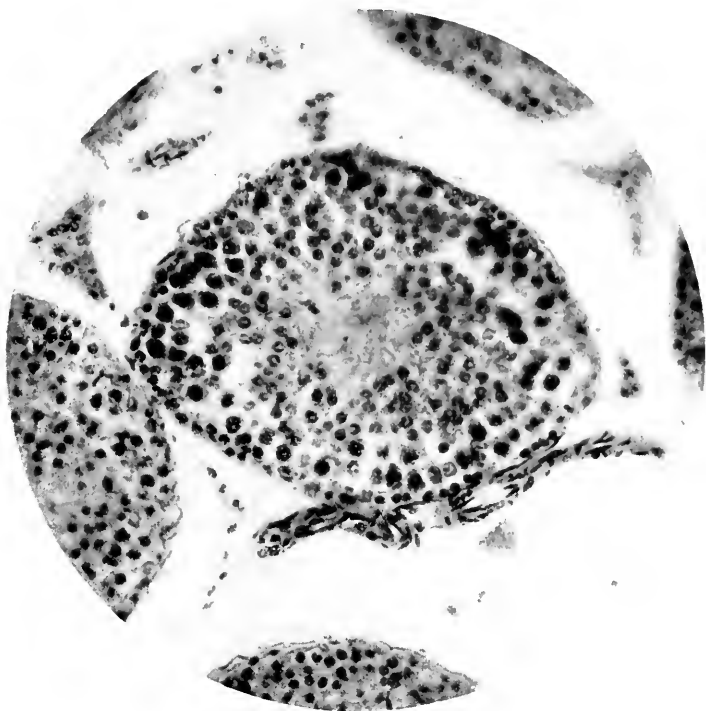


FIG. 5.

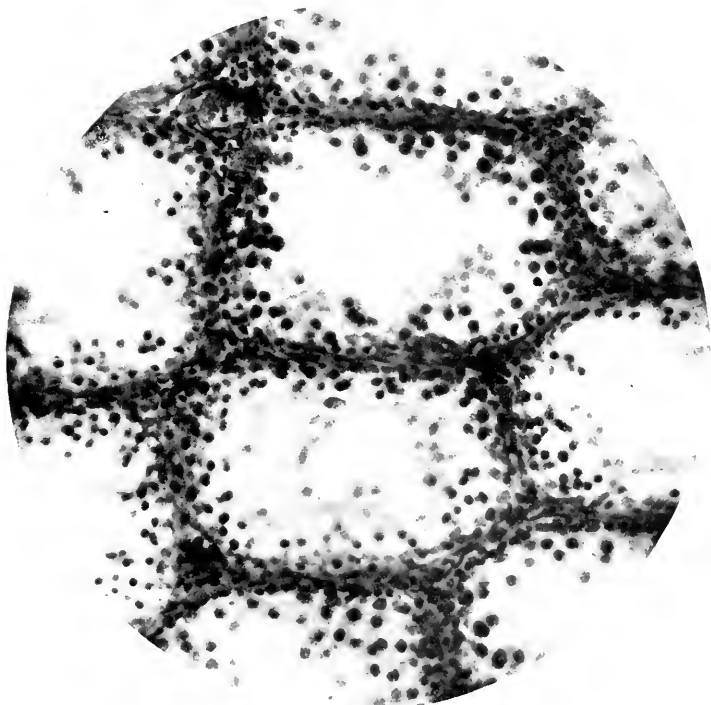
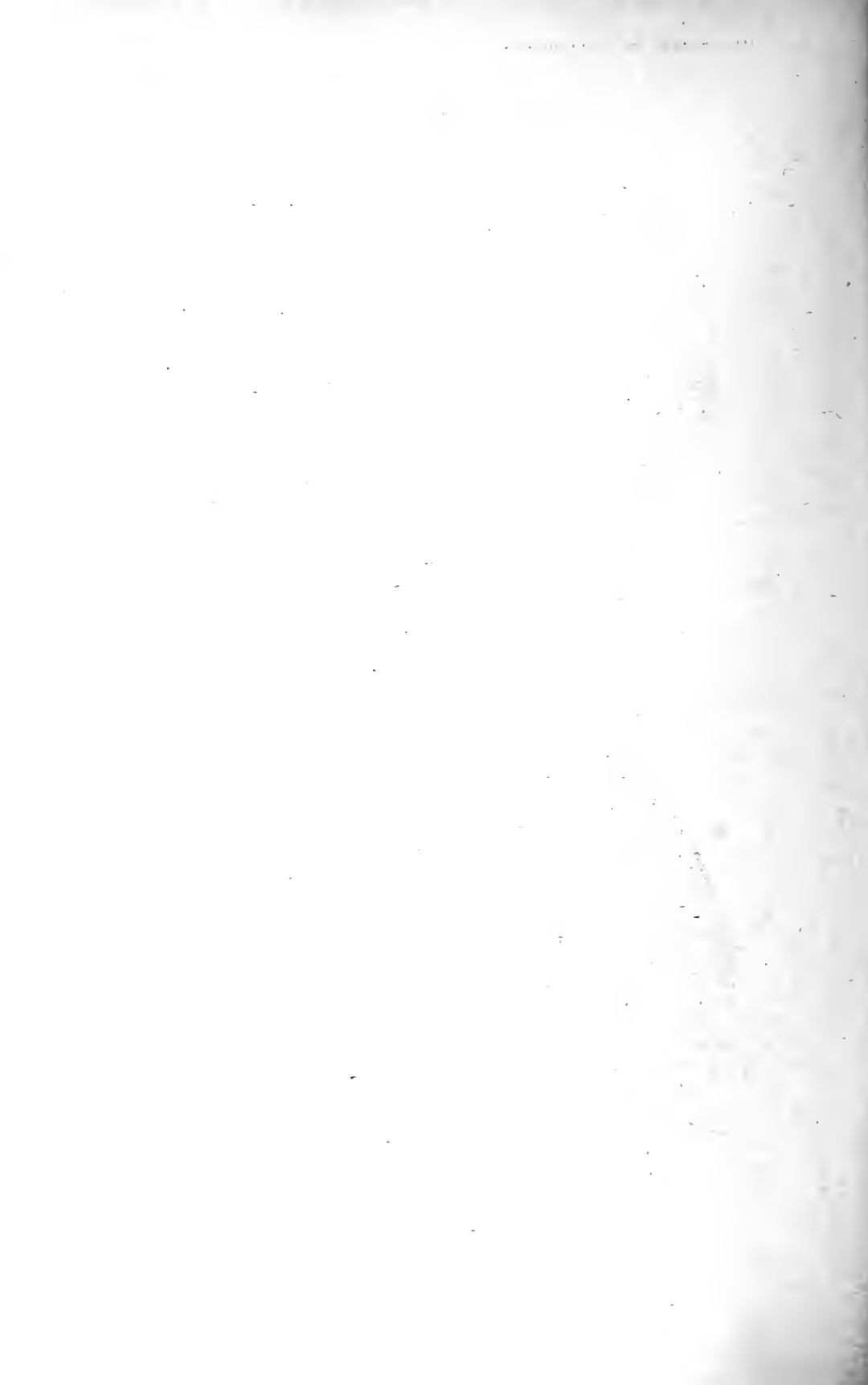


FIG. 6.

(Arlitt and Wells: Effect of alcohol on reproductive tissues.)



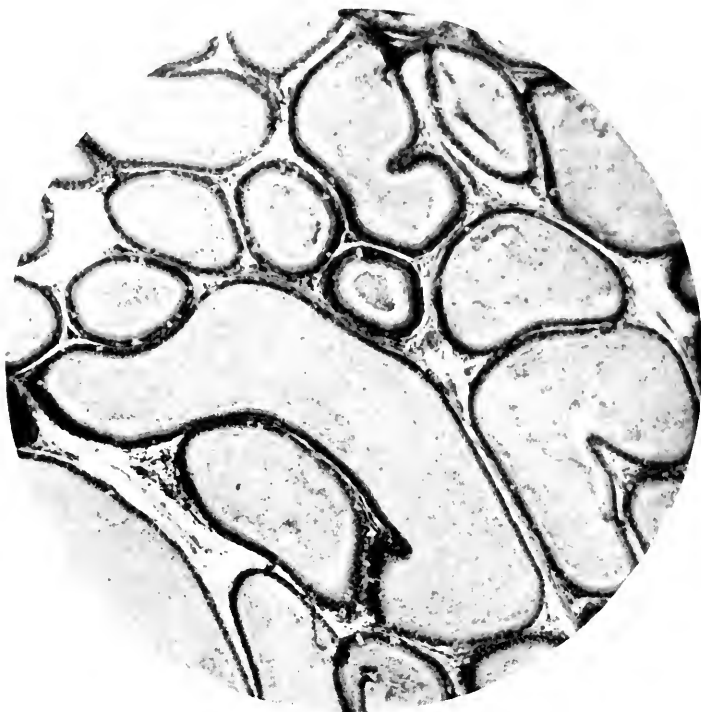


FIG. 7.

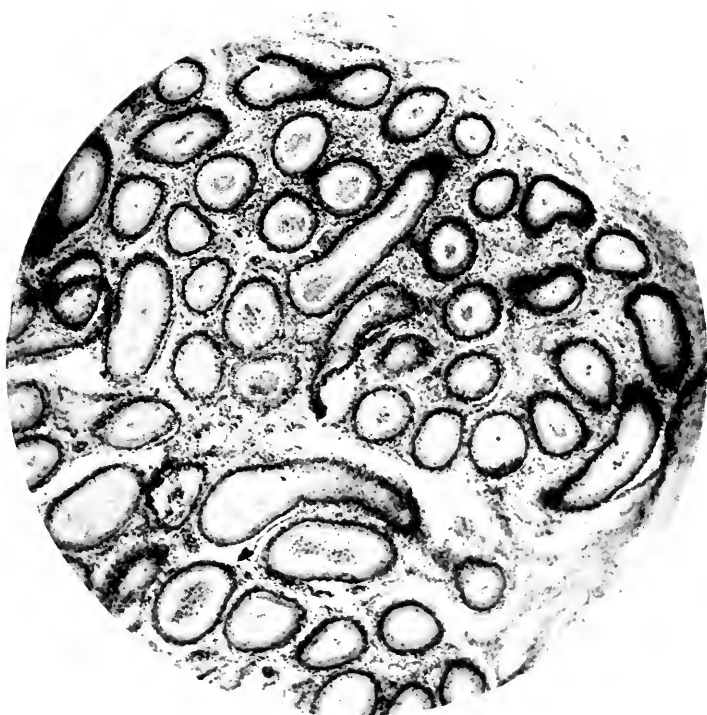


FIG. 8.

(Arlitt and Wells: Effect of alcohol on reproductive tissues.)



# MENINGITIS IN AN INFANT DUE TO A HITHERTO UNDESCRIBED ORGANISM, MICROCOCCUS FLORENS.

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PLATE 61.

(Received for publication, August 1, 1917.)

The clinical and bacteriological observations which we wish to record were made upon a case of meningitis which was admitted to the Harriet Lane Home of the Johns Hopkins Hospital. The clinical history was as follows:

H. S., age 6 months (Text-fig. 1). Dec. 15, 1916, 8 p.m. Admitted to the Harriet Lane Home in convulsions which had been continuous since 6 p.m. He had been treated in the dispensary for the preceding 3 weeks for otitis media. The temperature had been between 100.8° and 101.8° F. at each visit. Pus had been obtained from both ears by myringotomy.

The convulsions began suddenly on the day of admission. The child had vomited all of his feedings, was feverish, and looked very ill. On admission to the hospital he was found to be well developed and well nourished. He was having general convulsions, most marked in the left arm and leg. The eyes were fixed, staring, and deviated to the left. The convulsions were promptly controlled by sedatives. The physical examination was negative except for the double otitis media. The temperature was 102.4°F. There was no stiffness of the neck, no hyperesthesia, and Kernig's sign was negative. A lumbar puncture was done and a purulent fluid under increased tension obtained. The cells in the fluid were composed in large part of polymorphonuclear leukocytes with a few lymphocytes and several rather conspicuous, large mononuclear phagocytic cells. These became more numerous later in the disease. No organisms were demonstrable either in cultures or films. At the time of puncture, 15 cc. of the antimeningococcus serum of the Department of Health of the City of New York were given intraspinally. On the morning following admission the temperature had fallen to 100.3°F., but the child displayed frank evidences of meningitis, marked opisthotonus, painful rigidity of the neck, painful flexion

of the spine, positive Kernig sign, tache cérébrale, and marked hyperesthesia. The fontanelle was not bulging.

Because of the purulent fluid active serum therapy was continued twice daily in spite of the constant absence of organisms in films and cultures.

Dec. 18, 3rd day after admission. No residual signs of meningitis except slight cervical rigidity. The spinal fluid was clear and no serum was given.

Dec. 21, 7th day of the disease. The temperature rose to 102.5° F. and the child was irritable. The fluid was cloudy but no organisms could be demonstrated. Serum was again given and repeated on the following day.

The thirteenth puncture on the 13th day of the disease yielded fluid which was only slightly turbid. 4 days later, Dec. 31, it was perfectly clear with but 80 cells per c.mm.

Dec. 26-Jan. 3, 1917. Temperature normal; the patient seemed perfectly well.

Jan. 3, 20th day of the disease. There was a rise of temperature to 101.5° F., the child vomited in a projectile manner, became very irritable, and the neck again became stiff and retracted. The spinal fluid was cloudy and 12 cc. more of serum were given.

Jan. 6. The temperature again became normal and remained so for the 5 following days. The one lumbar puncture (No. 17) done during this time yielded almost clear fluid which contained 500 cells per c.mm., but no demonstrable organisms.

On the 29th day of the disease there was an abrupt rise of temperature to 102.8° F. and the child displayed again all the acute signs of meningitis. The eighteenth lumbar puncture gave very cloudy fluid in which, for the first time, organisms were demonstrable in great abundance both in films and cultures. They were Gram-negative intra- and extracellular diplococci, many of them arranged in groups of twos and threes. Serum was given then and for the 5 following days.

The fluid continued to be quite purulent, often as much as 1 cc. of pus settled in the bottom of a 6 cc. tube. Organisms continued to be present in the greatest abundance.

Jan. 14, 31st day. A ventricular puncture was done because it was impossible to withdraw the thick purulent material from the lumbar spine. Purulent fluid containing many organisms was obtained. Serum was given by the same route then and for the 2 days following.

Jan. 18. A Gram-negative diplococcus, identical with that found in the spinal fluid, was isolated from the blood stream.

The child's condition gradually became worse. He was stuporous, lying in a position of marked opisthotonus (Figs. 1 and 2). He paid no attention to his surroundings, but the fundi presented a normal appearance. The child was extremely hyperesthetic. His neck was very rigid, the extremities were quite spastic, and reflexes hyperactive. The fingers were tightly flexed at the wrist. It was necessary to pad the palms to prevent the finger nails from cutting into the palms.

Although food was well taken there were frequent attacks of projectile vomiting. There were no convulsions.

The opisthotonus increased until the soles of the feet almost touched the occiput. Fluid from the ventricle and lumbar spine 3 days before death was purulent and full of organisms.

Jan. 28, 45th day of the disease. The child died after the temperature had risen to 105.8° F. Autopsy was denied.

The bacteriology of the Gram-negative micrococcus repeatedly isolated from the spinal fluid of this case is of especial interest, inasmuch as the reactions of this organism do not correspond with those heretofore described by Weichselbaum and Ghon,<sup>1</sup> Jaeger,<sup>2</sup> Gordon,<sup>3</sup> Dopter and Pauron,<sup>4</sup> von Lingelsheim and Leuchs,<sup>5</sup> Shennan and Ritchie,<sup>6</sup> Elser and Huntoon,<sup>7</sup> and other investigators.<sup>8</sup> Because of its luxuriant growth on all media tested, we have tentatively called this organism *Micrococcus florens*.

As appears from Table I, this organism was isolated from four lumbar punctures, four ventricular punctures, and one blood culture. Three of these cultures were studied extensively; namely, one from the lumbar puncture on January 15, 1917, 13 days before death; one from the ventricular puncture on January 25, 3 days before

<sup>1</sup> Weichselbaum, A., *Fortschr. Med.*, 1887, v, 573; *Wein. klin. Woch.*, 1905, xviii, 992. Weichselbaum, A., and Ghon, A., *Wien. klin. Woch.*, 1905, xviii, 625.

<sup>2</sup> Jaeger, H., *Z. Hyg. u. Infektionskrankh.*, 1895, xix, 351; *Die Cerebrospinalmeningitis als Heeressuche*, Berlin, 1901; *Z. Hyg. u. Infektionskrankh.*, 1903, xlv, 225.

<sup>3</sup> Gordon, Great Britain National Health Insurance, Medical Research Commission, Special Report Series, No. 3, London, 1915-16.

<sup>4</sup> Dopter, C., *Compt. rend. Soc. biol.*, 1910, lxix, 546, 600; *Paris méd.*, 1911-12, ii, 211, 461. Dopter and Pauron, *Comp. rend. Soc. biol.*, 1914, lxxvii, 157, 231.

<sup>5</sup> von Lingelsheim, W., *Klin. Jahrb.*, 1906, xv, 373; *Z. Hyg. u. Infektionskrankh.*, 1908, lix, 457. von Lingelsheim and Leuchs, *Klin. Jahrb.*, 1906, xv, 489.

<sup>6</sup> Shennan, T., and Ritchie, W. T., *J. Path. and Bacteriol.*, 1908, xii, 456.

<sup>7</sup> Elser, W. J., and Huntoon, F. M., *J. Med. Research*, 1909, xx, 371.

<sup>8</sup> Arkwright, J. A., *J. Hyg.*, 1907, vii, 145; *Brit. Med. J.*, 1915, i, 494; *Proc. Roy. Soc. Med.*, 1914-15, viii, Sect. epidemiol. and state med., 69. Flexner, S., *J. Exp. Med.*, 1907, ix, 142; *Med. Rec.*, 1915, lxxxvii, 137. Wollstein, M., *J. Exp. Med.*, 1907, ix, 588. Du Bois, P. L., and Neal, J. B., *Am. J. Dis. Child.*, 1915, ix, 1; *J. Immunol.*, 1916, i, 307. Robinson, G. H., *J. Immunol.*, 1917, ii, 451 (also in *J. Am. Med. Assn.*, 1917, lxviii, 1660).

TABLE I.

Date.	Day of disease.	Temperature.		Signs of meningitis.	Lumbar puncture.	Ventricular puncture.	Character of fluid.	Organisms.		Amount of serum given.	Remarks.
		a.m.	p.m.					Films.	Cultures.		
1916										cc.	
Dec. 15	1	°F.	°F.	None.	1		Purulent.	0	0	15	White blood count 29,800.
"	2	100.3	101	Opisthotonus; positive Kernig sign; rigidity; hyperesthesia, etc.	2		"	0	0	10	
"	3	98.2	100	Cervical rigidity; Kernig.	3			0	0	10	
"	4	98.4	98.5	Slight cervical rigidity.	4			0	0	10	
"	5	99	100	Cervical rigidity; hyperesthesia.	5		Cloudy.	0	0	10	Von Pirquet sign negative.
"	6	102.5	102	"	6		Clear.	0	0	10	No tubercle bacilli found in films; globulin positive.
"	7	99	99	"	7		Turbid.	0	0	12	Nasal culture negative.
"	8	99.2	100.2	"	8		"	0	0	10	Urticaria.
"	9	99.6	100	Bulging fontanelle; spasmodic extremities.	9		600 cells per c.mm.; cloudy.	0	0	10	
"	10	98.6	98.2	Less marked.	10		Less cloudy.	0	0		Urine repeatedly negative.
"	11	98.6	98.6	None.	11		80 cells per c.mm.; clear.	0	0		Globulin faintly positive.
"	12				12						
"	13				13						
"	14				14						
"	15				15						
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1917	Jan. 3	20	99.3	101.5	Vomiting; stiff neck; hyperesthesia.	15	Purulent.	0	0	12	
"	4	21	101.4	102	"	16	"	0	0		Fundi normal.
"	5	22	100.4	100.8	"	17	"	0	0		Nasal culture negative.
"	8	25	98.8	98.6	None.		500 cells per c.mm.; almost clear.	0	0		
"	12	29	102.8	102.4	All.	18	Purulent.	++	++	10	Gold chloride gives atypical meningitic curve.
"	13	30	100.3	101.7	"	19	"	++	++	15	
"	14	31	101.3	101.8	"	20	"	++	++	10	
"	15	32	102	101	"	21	"	++	++	10	
"	16	33	101.9	101.8	"	22	"	++	++	10	
"	25	42	102.3	100	"	23	"	++	++	10	
						24	"	++	++	10	Jan. 18. Blood culture positive.
						25	"	++	++	Total serum given 189 cc.	

death; and the third obtained from a ventricular puncture made January 28, 5 hours after death. All the reactions of the Gram-negative micrococci obtained from these three punctures were constant. In the last puncture, in addition to this Gram-negative micrococcus, a Gram-positive coccus which subsequently proved to be *Staphylococcus albus* was obtained. Both the Gram-negative and Gram-positive cocci were found intra- and extracellular in the postmortem ventricular fluid.

Morphologically this organism is somewhat larger than the *Diplococcus intracellularis meningitidis* of Weichselbaum, although small forms were found in many of the cultures. It occurs chiefly as a diplococcus, although after subcultures single cocci, tetrads, and often larger groups of many cocci were found. No chains were demonstrable. In films the arrangement often resembled that of staphylococci.

Growth was luxuriant on the surface of 2 per cent agar, serum agar, blood agar, dextrose and other carbohydrate agars, hydrocele agar, and gelatin, and in beef broth and peptone water. On gelatin no growth occurred along the stab; the growth on the surface was luxuriant. No liquefaction was produced. No growth could be obtained beneath the surface of solid media. Growth on the various media was also luxuriant at room temperature. Agar cultures remained alive for more than 48 days.

Single colonies on agar plates at the end of 16 hours were round and moist, with a smooth and uniformly elevated surface, and measured 1.5 mm. in diameter. They were grayish white in color. At the end of 4 or 5 days they became a dirty grayish brown.

Table II shows the fermentation reactions of this organism (*Micrococcus florens*), *Bacillus typhosus*, *Micrococcus catarrhalis*, and *Bacillus faecalis alkaligenes* grown on media from the same batch. The reactions were constant on all the media used; namely, beef broth, peptone water, and 2 per cent agar, containing 1 per cent of the various carbohydrates and 0.1 per cent of Andrade's indicator.<sup>9</sup>

*Micrococcus florens* did not produce gas in any media, but formed acid in arabinose, dextrose, and galactose. In the agar slant cultures no growth occurred below the surface.

<sup>9</sup> Andrade, E., *J. Med. Research*, 1905-06, xiv, 551.

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TABLE II.

Organism.	Glycerol.	Ara binose.	Dextrose.	Galactose.	Mannite.	Levulose.	Lactose.	Maltose.	Saccharose.	Raffinose.	Dextrin.	Inulin.	Salticn.
<i>M. florens</i> *	0	+ (24 hrs.)	+ (24 hrs.)	+ (24 hrs.)	0	0	0	0	0	0	0	0	0
<i>M. catarrhalis</i> .....		0	0	0									
<i>B. faecalis alkaligenes</i>			0	0	0	0	0	0	0	0	0		
<i>B. typhosus</i> .....			+ (24 hrs.)	+ (24 hrs.)	+ (24 hrs.)	+ (24 hrs.)	+ (24 hrs.)	+ (24 hrs.)	0	0	+ (24 hrs.)		

\*These fermentation reactions were tested twelve times by two different bacteriologists. Carbohydrates of two different lots were used. Tests were incubated at 37°C. for 30 days and read daily.

The reactions of *Micrococcus florens* with dextrose and arabinose on solid media were slightly less marked than in the fluid media, but that for galactose was equally well marked on solid and in fluid media.

The media were made and sterilized in the following way: Beef broth, peptone water, and 2 per cent agar were made according to the directions recommended by Hiss and Zinsser.<sup>10</sup> To each of the different series 1 per cent of one of the thirteen carbohydrates was added. Merck's pure carbohydrates were used in these tests. Andrade's indicator was then added up to 0.1 per cent. A small amount of this mixture was measured into a tube, warmed, and the reaction was made neutral with normal sodium hydroxide; *i.e.*, just pink while hot. The amount of normal sodium hydroxide calculated to make the total volume of each series neutral was then added to each lot and the different series were tubed. The media were colorless when cool.

They were sterilized by placing them in an Arnold sterilizer which had first been heated to 100°C. The tubes were sterilized at this temperature for 20 minutes on 3 consecutive days. They were then incubated for 48 hours at 37°C. and proved sterile.

That this method of manufacture and sterilization of the media did not break down the various carbohydrates is demonstrated by the fact that on media of the same batch *Bacillus typhosus*, *Micrococcus catarrhalis*, and *Bacillus faecalis alkaligenes* gave their characteristic fermentation reactions.

#### *Serological Reactions.*

The agglutination method used in these tests was that introduced by Dreyer<sup>11</sup> in 1904, with the exception that instead of the formalized standardized cultures used by Dreyer, living 24 hour beef broth cultures were employed except for the normal and para meningococci. For the normal and para meningococci a saline emulsion of a 24 hour serum agar slant culture was used. The reason for the

<sup>10</sup> Hiss, P. H., and Zinsser, H., A text-book of bacteriology, New York, 3rd edition, 1916.

<sup>11</sup> Dreyer, G., *J. Path. and Bacteriol.*, 1909, xiii, 331 (also Davison, W. C., *J. Am. Med. Assn.*, 1916, lxxvi, 1297; *J. Lab. and Clin. Med.*, 1917, ii, 607).

latter exception was the difficulty of growing meningococci in beef broth.

The opacities of all these cultures, both saline emulsions and broth cultures, were made approximately equal, so that the results of these tests may be compared with each other.

All the agglutination tests were repeated several times on different days, and with the exception of a progressive diminution in titer of the patient's serum and spinal fluid due to age, the results of all the tests were constant. Tables III to VII show characteristic agglutination tests made on the same day.

Table III shows the results of agglutination tests made with the patient's serum, obtained 5 hours after death, and living 24 hour beef

TABLE III.

Serum.	Organism.	Dilutions of serum.				Control.
		1:25	1:50	1:125	1:250	
Patient's serum.	<i>M. florens.</i>	T.*	T.	Tr.	0	0
" "	<i>M. catarrhalis.</i>	0	0	0	0	0
" "	Normal meningococcus.	0	0	0	0	0
" "	Para "	0	0	0	0	0

\* In the tables T. indicates total, or complete agglutination, the supernatant fluid being absolutely clear and all the bacteria being in the sediment at the bottom of the tube; S., standard, or medium sized flocculi in the fluid without sedimentation; and Tr., trace, or agglutination just visible to the naked eye.

broth cultures of *Micrococcus florens* and *catarrhalis*, and saline emulsions of normal and para meningococci.<sup>12</sup> The patient's serum agglutinated the organism we have isolated and did not affect *Micrococcus catarrhalis* or the normal or para meningococcus.

Table IV shows the results of agglutinative tests made with the patient's ventricular fluid, obtained 5 hours after death, and *Micrococcus florens*, *Micrococcus catarrhalis*, and normal and para meningococci. The results of this test are similar to, though with a lower titer than those obtained with the patient's serum.

<sup>12</sup> We are indebted to Dr. Harold L. Amoss and Dr. Wilson G. Smillie of The Rockefeller Institute for Medical Research for sending us these cultures of meningococci.

Table V shows the result of similar agglutination tests made with the serum from a rabbit before and after immunization with two intravenous doses of a saline emulsion of agar slants of *Micrococcus florens*. This serum, after immunization, only agglutinated cultures of *Micrococcus florens* and did not affect either *Micrococcus catarrhalis*, *Staphylococcus albus*, or the normal or para meningococcus.

Table VI shows the results of agglutination tests made with the polyvalent antimeningitis serum issued by the Department of

TABLE IV.

Serum.	Organism.	Dilutions of serum.				Control.
		1:25	1:50	1:125	1:250	
Patient's ventricular fluid.	<i>M. florens</i> .	Tr.	0	0	0	0
" " "	<i>M. catarrhalis</i> .	0	0	0	0	0
" " "	Normal meningococcus.	0	0	0	0	0
" " "	Para "	0	0	0	0	0

TABLE V.

Serum.	Organism.	Dilutions of serum.							Control.
		1:25	1:50	1:125	1:250	1:500	1:1,250	1:2,500	
Rabbit serum before immunization.	<i>M. florens</i> .	0	0	0	0	0	0	0	0
" " after "	<i>M. "</i>	T.	T.	T.	T.	Tr.	Tr.	Tr.	0
" " " "	<i>M. catarrhalis</i> .	0	0	0	0	0	0	0	0
" " " "	Normal meningococcus.	0	0	0	0	0	0	0	0
" " " "	Para "	0	0	0	0	0	0	0	0
" " " "	<i>S. albus</i> .	0	0	0	0	0	0	0	0

Health of the City of New York and *Micrococcus florens*, *Micrococcus catarrhalis*, and the normal and para meningococcus. This serum agglutinated only the normal and para meningococcus emulsion and did not affect the organism of our case (*Micrococcus florens*) or *Micrococcus catarrhalis*.

Table VII shows the result of an agglutination test made with The Rockefeller Institute polyvalent antimeningitis serum and *Micrococcus florens*, *Micrococcus catarrhalis*, and the normal and para



meningococcus. The results in Table VII are similar to those in Table VI.

Table VIII shows the results of autolysis experiments.<sup>13</sup> The growth from 16 hour sheep serum agar slant cultures of each organism was suspended in 10 cc. of normal saline solution, seven drops of toluene being added to each tube and the tube well shaken. The tubes were incubated and examined at the end of 4 and 6 hours.

TABLE VI.

Serum.	Organism.	Dilutions of serum.					Control.
		1:25	1:50	1:125	1:250	1:500	
Polyvalent antimenigitis serum (Department of Health, New York City)	<i>M. florens.</i>	0	0	0	0	0	0
" "	<i>M. catarrhalis.</i>	0	0	0	0	0	0
" "	Normal meningococcus.	T.	T.	S.	Tr.	0	0
" "	Para "	"	"	T.	"	0	0

TABLE VII.

Serum.	Organism.	Dilutions of serum.							Control.
		1:25	1:50	1:125	1:250	1:500	1:1,250	1:2,500	
Polyvalent antimenigitis se- rum (Rockefeller Institute).	<i>M. florens.</i>	0	0	0	0	0	0	0	0
" "	<i>M. catarrhalis.</i>	0	0	0	0	0	0	0	0
" "	Normal meningococcus.	T.	T.	T.	T.	Tr.	0	0	0
" "	Para "	"	"	"	"	S.	Tr.	0	0

As will be seen from Table VIII *Micrococcus florens* does not autolyze as readily as the normal and para meningococcus, while *Micrococcus flavus* 3 autolyzes still less than *Micrococcus florens*.

<sup>13</sup> These tests were kindly made by Dr. Wilson G. Smillie of The Rockefeller Institute for Medical Research.

TABLE VIII.

Organism.	Degree of autolysis.	
	4 hrs.	6 hrs.
Normal meningococcus (Rockefeller Institute)	+++	Almost complete.
Para " " "	++	+++
<i>M. florens</i> .....	+-	+
<i>M. flavus</i> 3.....	-	-

*Pathogenicity Tests.*

*Mice*.—(a) and (b). The pathogenicity of *M. florens* for mice was tested on two animals. 1.0 cc. of a saline emulsion containing one-tenth of a living 24 hour agar slant of this organism when injected into the peritoneums of two white mice produced death in 11 to 18 hours in each instance.

Films made of the milky peritoneal exudate and stained with Hiss' capsular stain<sup>10</sup> failed to show any capsules. *M. florens* was recovered in cultures, made at the autopsies, of the heart's blood and of the peritoneal exudate.

*Rabbits*.—The pathogenicity of *Micrococcus florens* for rabbits was tested on four animals.

(a) 1.5 cc. of a saline emulsion containing one-sixth of a living 24 hour agar slant of the organism injected into the lumbar cord<sup>14</sup> of Rabbit 1 failed to cause any symptoms for 15 days. On the 15th day 1.75 cc. of a saline emulsion containing one-fifth of a living 24 hour agar slant of the organism were again injected into the lumbar cord. This injection produced death within 12 hours.

The autopsy showed slight thickening of the meninges, enlargement of the spleen, and marked injection of the blood vessels of the intestine and mesentery. Histological sections of the brain and spinal cord showed the presence of meningitis. Cultures of the spinal cord and the heart's blood, made at autopsy, showed the presence of *M. florens*.

Table IX shows the result of agglutination tests made with serum from this rabbit, obtained immediately before its first inoculation, and on the 15th day, immediately before the second inoculation. The production of agglutinins for *Micrococcus florens* is demonstrated.

(b) 0.5 cc. of a saline emulsion containing one-twentieth of a living 24 hour agar slant of *M. florens* was injected intracerebrally (left frontal lobe) into Rabbit 2. No symptoms were noted for 10 days. At the end of that time, 0.5 cc. of a

<sup>14</sup> The technique for lumbar puncture for rabbits is that employed by C. R. Austrian (*Bull. Johns Hopkins Hosp.*, 1916, xxvii, 237).

saline emulsion containing one-fifth of a living 24 hour agar slant of *M. florens* was again injected intracerebrally.

A culture of the heart's blood, made by aseptic cardiopuncture 24 hours after the last inoculation, showed a growth of *M. florens*. A progressive weakness and general malaise were noted for the next 10 days, at the end of which death occurred.

*M. florens* was recovered from cultures made at autopsy from the brain and spinal cord. Histological sections of the brain and spinal cord showed evidence of a chronic meningitis.

(c) and (d). 1.0 cc. of a saline emulsion containing one-fourth of a living 24 hour agar slant of *M. florens* was injected into the lower lumbar cords of Rabbits 3 and 4. No. 3 died in 8 to 14 hours. No. 4 died in 12 to 18 hours.

*M. florens* was recovered from cultures made at autopsy of the heart's blood and the spinal cord of each rabbit. The rabbits' sera before inoculation and after death did not agglutinate *M. florens*. Microscopic sections of the spinal cords showed the presence of an acute purulent meningitis in each animal.

TABLE IX.

Serum.	Organism.	Dilutions of serum.				Control.
		1:25	1:50	1:125	1:250	
Rabbit 1 (1st day) before first inoculation.....	<i>M. florens</i> .	0	0	0	0	0
" 1 (15th " ) " second " .....	<i>M. "</i>	T.	Tr.	0	0	0

*Monkeys.*—(a) 0.5 cc. of saline emulsion containing one-fifth of a living 24 hour agar slant of *M. florens* was inoculated into the left frontal lobe of a monkey. This monkey, a *Macacus rhesus*, was obviously suffering from miliary tuberculosis at the time. For the next 15 hours no symptoms of meningitis could be noted. Cultures of the heart's blood, obtained by cardiopuncture, at the end of this time revealed a growth of *M. florens*.

At the end of 24 hours 0.5 cc. of a saline emulsion containing one-fifth of a living 24 hour culture of *M. florens* was again injected intracerebrally, into the left frontal lobe. At the end of 12 hours the monkey was decidedly ill and very irritable, lying on the bottom of his cage, refusing food. No opisthotonus could be noted although the monkey evidenced pain when his neck was bent. Symptoms became more marked and death followed in 60 hours after the second inoculation.

The autopsy showed miliary tuberculosis involving the mediastinal and mesenteric glands, the great omentum, the kidneys, the spleen, and the body of the tenth thoracic vertebra. No tuberculous processes were noted in the central nervous system. Films of the brain and spinal cord showed large numbers of polymorphonuclear leukocytes and Gram-negative diplococci. There were ad-

hesions between the frontal and temporal lobes. Histological sections of the brain and spinal cord gave obvious evidence of acute purulent meningitis. Cultures made of the heart's blood, brain, and spinal cord showed growths of *M. florens*.

Table X shows the results of agglutination tests made with this monkey's serum before the first inoculation and at autopsy.

(b) 1.5 cc. of a saline emulsion containing one-quarter of the growth from one 24 hour agar slant culture of *M. florens* were injected into the lower lumbar

TABLE X.

Serum.	Organism.	Dilutions of serum.				Control.
		1:25	1:50	1:125	1:250	
Monkey 1, before first inoculation.....	<i>M. florens</i> .	0	0	0	0	0
“ 1, at autopsy (4 days later).....	<i>M. “</i>	T.	T.	T.	T.	0

TABLE XI.

Serum.	Organism.	Dilutions of serum.				Control.
		1:25	1:50	1:125	1:250	
Monkey 2, before first inoculation.....	<i>M. florens</i> .	0	0	0	0	0
“ 2, “ third “ (22 days later)....	<i>M. “</i>	T.	T.	T.	T.	0
“ 2, at autopsy (24th day).....	<i>M. “</i>	“	“	“	“	0

cord of a healthy *Macacus rhesus* (Monkey 2). No symptoms were noted for the next 3 days. At the end of this time 1.5 cc. of a saline emulsion containing one-half of the growth from a 24 hour agar slant culture were injected into the lower lumbar cord. No symptoms occurred for the next 19 days. At the end of this time 1.75 cc. of a saline emulsion containing all the growth from one 24 hour agar slant of *M. florens* were again injected into the lower lumbar cord. Death resulted in 8 to 18 hours.

*M. florens* was recovered in cultures made at autopsy from the heart's blood, brain, and spinal cord. Histological sections of the brain and spinal cord showed evidence of acute purulent meningitis. No other lesions could be found.

Table XI shows the results of agglutination tests made with this monkey's serum before the first inoculation, before the third inoculation (22 days after the first and 19 days after the second inoculation), and after death.

## CONCLUSIONS.

These virulence tests lead us to believe that *Micrococcus florens* is pathogenic for mice, rabbits, and monkeys (*Macacus rhesus*), producing septicemia, and that it will also cause purulent meningitis in the two latter animals.

The combined results of the biological reactions and of the agglutination tests lead us to believe that we are dealing with an organism which, though morphologically similar to the meningococcus, yet is entirely distinct.

A review of the literature fails to reveal any description of a Gram-negative micrococcus which grows luxuriantly on all media tested, forms no pigment, and ferments arabinose, dextrose, and galactose.

We wish to express our thanks to Dr. Anna Williams and to Dr. Stanhope Bayne-Jones for their valuable suggestions and assistance.

## EXPLANATION OF PLATE 61.

FIGS. 1 and 2. These photographs were taken 3 days before death, showing the child in different positions. The opisthotonus was extreme.





FIG. 1.



FIG. 2.

(Davison, Davison, and Miller: Meningitis due to *Micrococcus florens*.)





## THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

### XVI. THE INFLUENCE OF SPLENECTOMY AND OF BLOOD DISINTEGRATION UPON THE PRODUCTION OF BILE PIGMENT.

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(Received for publication, July 9, 1917.)

In previous studies (1) reported from this laboratory the various phenomena which follow splenectomy in the dog have been described. In connection with the most important of these—the anemia, the increased resistance of the red cells, and the decreased tendency to jaundice—numerous indirect observations indicated that in the absence of the spleen, there is possibly a decreased formation of bile pigment. In the present work this point was studied by a direct examination of the pigment content of the bile.

Banti (2) was the first to point out that splenectomized dogs require larger doses of toluylenediamine to cause jaundice than do normal dogs. Pugliese and Luzzatti (3) confirmed this observation and brought evidence to show that the action of toluylenediamine and pyrodine upon the splenectomized animal is less marked and occurs less promptly than in the normal animal. Joannovics (4) agreed in this general opinion and claimed, as did also Vast (5), that for the destruction of the blood cells by toluylenediamine the intervention of the spleen is necessary. The only direct observations appear to be those of Pugliese (6) who states that after splenectomy the output of bile pigment in bile from a bile fistula drops to about one-half, while the other constituents of the bile are but little altered. Moreover, he showed that although after the injection of pyrodine the bile of the splenectomized dogs shows an increase in the bile pigments, the increase is not so pronounced as in the normal dog after pyrodine injection.

The recent work of Hooper and Whipple (7 *c, d, e, f, h*) does not support this claim of Pugliese. On the contrary, these observers assert that splenectomy has no constant influence on the output of bile pigments under normal conditions, though they find many interesting deviations from the normal output in splenectomized bile fistula dogs with anemia.

*Methods.*

The elimination of bile pigment and the changes following the administration of an hemolytic agent were studied both before and after splenectomy in dogs with a bile duct-ureter anastomosis (8); at the same time the studies were made of the red cell and hemoglobin content of the blood.

Four dogs of approximately the same size were used. They were fed on a standard diet containing 0.4 gm. of nitrogen per kilo and 70 calories per kilo of body weight. The diet consisted of beef heart, lard, bread crumbs, sugar, a little salt, and some bone ash. The daily intake of water was always the same.

The importance of keeping the test animals on a constant diet for bile pigment determination is obvious from the observations of Hooper and Whipple (7 *b*), who have shown that carbohydrates tend to increase considerably the secretion of bile pigment in dogs with bile fistula and also that on a strict meat diet the bile pigment curve is at its lowest level.

Instead of the usual external bile fistula, the bile duct-ureter fistula (or anastomosis) of Pearce and Eisenbrey (8) was employed. The method consists in diverting the bile from the intestine to the urinary bladder by anastomosing the common bile duct and the right ureter, after removal of the corresponding kidney. It has several advantages for long continued observations and avoids some of the troubles of the external fistula, such as obstruction, infection, and losses due to accidents in collecting. The mixture of urine and bile, in the absence of jaundice, offers no difficulties for the quantitative determination of bile pigments.<sup>1</sup>

Several days after this operation a preliminary blood examination was made and if anemia was absent, and there was no evidence of obstruction of flow of bile to the bladder, the urine was collected from the cage every morning for 1 week and the several lots were combined for quantitative estimation of bile pigment.

The animals were not catheterized, the urine being collected daily

<sup>1</sup> I am indebted to Professor J. E. Sweet of the Department of Surgical Research for the performance of these operations.

and preserved in cold storage until the quantitative determination of bile pigment was made.

It is well known that there are hourly and at times daily variations in bile pigment output (7 *a*, 9), and it was to avoid errors due to these variations that collections were continued for so long a time. This control period we hereafter refer to as Period A.

In the second period, of equal duration with A, Period B, an hemolytic agent, toluylenediamine, was given. This was made up in a 2 per cent solution and administered by stomach tube daily at the same hour for 7 days.

After Period B had been completed, an interval of more than 10 days was allowed to elapse to permit recovery from the anemia due to toluylenediamine. Splenectomy was then performed under ether anesthesia. 1 week after this operation, observations similar to those of Period A were made. This period is known as Period C.

In Period D, toluylenediamine was given as in Period B. In each period observations were made of the condition of the blood and of the weight and general condition. At the termination of each experiment the anastomosis, the bile passages, liver, ureter, and urinary bladder were carefully examined. No abnormalities were found except a slight dilatation of the bile ducts with slight bile stasis in the liver in Dog 2.

In the first experiment (Dog 1) two observations were made in Period A, and in the first and second (Dogs 1 and 2) two sets in Period B. In the other animals, only one set was made in all periods.

For the determinations of the amount of bile pigment in the urine, the method of Hooper and Whipple (7*a*) was used. Exactly 10 cc. of bile-urine mixture were placed in a large glass tube, 20 cc. of 10 per cent calcium chloride solution were added; the mixture was then made weakly alkaline with a saturated solution of sodium carbonate and centrifuged for 30 minutes at a speed of 1,500 revolutions per minute. After this, a small amount of calcium chloride solution was added to see whether precipitation would occur again. After complete precipitation was assured, the precipitate was washed four times with distilled water by use of the centrifuge. To dissolve the precipitate finally, the following solution was used:

		cc.
Ethyl alcohol,	95 per cent.....	100
Nitric acid,	50 " " .....	0.4
Hydrochloric acid,	37 " " .....	5.0

After standing for 16 to 18 hours, when the intensity of color was at its maximum, the pigment was estimated by the Duboscq colorimeter. The standardization of the wedge in the colorimeter was made against Kahlbaum's chemically pure bilirubin.

For determining the hemoglobin content of the blood the Fleischl-Miescher apparatus was used.

The resistance of the erythrocytes was examined by hypotonic salt solution; there was very slight decrease after the administration of toluylenediamine and very slight increase after the splenectomy.

#### RESULTS.

The general outcome of these experiments is shown in Tables I to IV. In the first period the general condition of the animal—weight and blood picture—was satisfactory. In Period B the blood changes due to toluylenediamine were accompanied by a slight loss of weight and this was evident also in Period D, but in general the changes were not sufficient to prevent comparable observations in the various periods. It should be emphasized that in the four experiments reproduced in tabular form, there was no loss of bile through faulty anastomosis and no evidence of jaundice due to obstruction. In studying the tables Period A should be compared with Period C and Period B with Period D, the former showing the effect of splenectomy alone and the latter of splenectomy on the formation of bile pigment in the presence of excessive blood destruction. This comparison shows that in each experiment the elimination of bile pigment after splenectomy was reduced and the observations are thus in accord with those of Austin and Pepper (1 *l*), which indicated that the path of the blood to the liver is an important factor in the production of jaundice. There is, however, another possible factor. Pearce, Austin, and Musser (1 *c*) have shown that the production of jaundice is more difficult in an anemic animal than in the plethoric animal, and Hooper and Whipple (7 *g*), who found that splenectomy did not influence the output of bile pigment in the dog after several months following splenectomy, describe many curious results in the anemic splenectomized animal. In all the experiments the blood picture was never so satisfactory in Period C as in Period A and the factors

of anemia and blood regeneration undoubtedly have something to do with the results we obtained. There is, however, no question, under the condition of these experiments, of the decreased elimination of bile pigment after splenectomy. In this connection it should be pointed out that the periods of continuous observation—7 days—are larger

TABLE I.

Dog 1. Weight 9.3 kilos.

Put on diet\* January 15, 1917.

Bile duct-ureter anastomosis January 19.

Period.	Dose of 2 per cent toluylenediamine.	Bile pig- ment per day.	Blood.			Weight.
			Date.	Hemo- globin.	Erythrocytes per cm.	
<i>1917</i>		<i>gm.</i>	<i>1917</i>	<i>per cent</i>		<i>kg.</i>
Period A.						
Jan. 23-29		0.0678	Jan. 23	98	6,210,000	9.5
" 30-Feb. 5		0.0644	" 30	100	6,030,000	9.4
Period B.						
Feb. 6-12	10 cc. every day.	0.0698	Feb. 8	82	5,450,000	9.0
			" 13	94	5,700,000	7.8
" 21-27	13 cc. on Feb. 21, 22, 23; 15 cc. on Feb. 24, 25, 26, 27.	0.310	" 19	103	5,170,000	8.0
			" 23	95	5,200,000	8.0
			" 27	43	4,190,000	7.8
Mar. 16. Splenectomy.			Mar. 15	101		
Period C.						
Mar. 22-28		0.0450	Mar. 22	89	5,300,000	8.2
			" 28	82	5,360,000	8.1
Period D.						
Mar. 29-Apr. 4	13 cc. on Mar. 29, 30, 31; 15 cc. on Apr. 1, 2, 3, 4.	0.208	Mar. 30	74	5,230,000	7.5
			Apr. 4	63	3,600,000	7.5

\* Diet consisted of beef heart 150 gm., lard 30 gm., sugar 40 gm., bread crumbs 50 gm., bone ash 10 gm., salt 2 gm., and water 300 cc.

than those of other observers and thus obviate error due to temporary fluctuations—possibly a source of error in shorter periods of observations.

A comparison of Periods A and B shows that small doses over several days of an hemolytic poison may not cause an increase in bile pigment elimination, but if continued in larger doses or for a longer period a

fivefold increase in elimination may occur (Table I). This is in accord with the views of Pearce, Austin, and Eisenbrey (1 *b*) on the relation of hemoglobinemia to jaundice.

If Periods B and D are compared, it is seen that although in Period D an increase of pigment elimination follows blood destruc-

TABLE II.

Dog 2. Weight 10.5 kilos.

Put on diet\* January 20, 1917.

Bile duct-ureter anastomosis January 23.

Period.	Dose of 2 per cent toluylenediamine.	Bile pigment per day.	Blood.			Weight.
			Date.	Hemo- globin.	Erythrocytes per cm.	
<i>1917</i>		<i>gm.</i>	<i>1917</i>	<i>per cent</i>		<i>kg.</i>
Period A. Jan. 30-Feb. 5		0.0622	Feb. 5	101	5,680,000	9.9
Period B. Feb. 6-12	10 cc. every day.	0.0672	Feb. 8	68	3,950,000	9.4
			" 13	71	4,010,000	8.9
			" 19	86	5,480,000	
" 21-27	13 cc. on Feb. 21, 22, 23; 15 cc. on Feb. 24, 25, 26, 27.	0.1280	" 23	52	3,550,000	8.9
			" 27	38	3,520,000	8.6
Mar. 16. Splenectomy.			Mar. 15	99		
Period C. Mar. 22-28		0.036	Mar. 22	81	4,460,000	8.9
			" 28	82	4,280,000	8.2
Period D. Mar. 29-Apr. 4	13 cc. on Mar. 29, 30, 31; 15 cc. on Apr. 1, 2, 3, 4.	0.103	Mar. 30	62	3,510,000	8.0
			Apr. 4	35	3,140,000	7.9

\* Diet consisted of beef heart 150 gm., lard 40 gm., sugar 40 gm., bread crumbs 50 gm., bone ash 10 gm., salt 2 gm., and water 300 cc.

tion, this increase does not reach the high level obtained before splenectomy (Period B). There is here no question of the influence of anemia, as was the case in comparing Periods A and C, for in all experiments in Periods B and D anemia was present, and was of approximately the same degree. It is difficult therefore to avoid the conclusion that the lowered elimination of bile pigment is due to the absence of the spleen.

TABLE III.

Dog 3. Weight 8.6 kilos.

Put on diet\* February 27, 1917.

Bile duct-ureter anastomosis March 1.

Period.	Dose of 2 per cent toluylenediamine.	Bile pigment per day.	Blood.			Weight
			Date.	Hemo- globin.	Erythrocytes per cm.	
<i>1917</i>		<i>gm.</i>	<i>1917</i>	<i>per cent</i>		<i>kg.</i>
Period A. Mar. 5-11		0.0624	Mar. 6	102	6,120,000	8.6
Period B. Mar. 12-18	14 cc. every day.	0.288	Mar. 13 " 18	82 45	3,930,000 3,420,000	8.4 7.6
Mar. 30. Splenectomy.			Mar. 29	100		
Period C. Apr. 5-11		0.0396	Apr. 6 " 11	82 64	5,120,000 4,860,000	8.4 7.8
Period D. Apr. 12-18	14 cc. every day.	0.202	Apr. 13 " 18	58 40	3,570,000 3,240,000	7.5 7.3

\* Diet consisted of beef heart 110 gm., lard 35 gm., sugar 35 gm., bread crumbs 30 gm., bone ash 10 gm., salt 2 gm., and water 300 cc.

TABLE IV.

Dog 4. Weight 8.4 kilos.

Put on diet \* February 27, 1917.

Bile duct-ureter anastomosis March 1.

Period.	Dose of 2 per cent toluylenediamine.	Bile pigment per day.	Blood.			Weight.
			Date.	Hemo- globin.	Erythrocytes per cm.	
<i>1917</i>		<i>gm.</i>	<i>1917</i>	<i>per cent</i>		<i>kg.</i>
Period A. Mar. 5-11		0.0618	Mar. 6	94	5,980,000	8.2
Period B. Mar. 12-18	14 cc. every day.	0.186	Mar. 13 " 18	77 42	4,360,000 3,433,000	8.1 7.4
Mar. 30. Splenectomy.			Mar. 29	90		
Period C. Apr. 5-11		0.036	Apr. 6 " 11	84 69	4,820,000 4,250,000	8.1 7.5
Period D. Apr. 12-18	14 cc. every day.	0.1256	Apr. 13 " 18	61 38	4,060,000 3,220,000	7.0 7.2

\* Diet consisted of beef heart 110 gm., lard 35 gm., sugar 35 gm., bread crumbs 30 gm., bone ash 10 gm., salt 2 gm., and water 300 cc.

## SUMMARY.

1. In four animals with a bile duct-ureter anastomosis and without disturbance due to obstruction or absorption, the total quantity of bile pigment output during a day under normal conditions varied from 0.0618 to 0.0678 gm. These figures are practically identical with those of Stadelmann (9, 10) but lower than those given by Hooper and Whipple (7), who find that the average bile pigment excretion amounts to about 1 mg. per pound of body weight per 6 hours.

2. In all the experiments there is definite evidence of a decrease in bile pigment elimination after splenectomy. This is true not only of the elimination when no hemolytic agent is administered but also when excessive blood destruction is caused. Under the latter circumstances the amount of bile pigment is greatly increased but never reaches the high level of blood destruction before splenectomy.

3. These observations appear to show conclusively that the absence of the spleen influences the formation of bile pigment. To what extent the influence is mechanical, *i.e.*, change in the course of the blood to the liver, and to what extent due to anemia, cannot be stated at present.

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## STAGGERS IN SHEEP IN PATAGONIA.\*

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PLATES 62 TO 65.

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### INTRODUCTION.

During the past few years a nervous disorder of sheep has become more prevalent throughout portions of Patagonia. This disorder has been given a number of names; among the more common may be mentioned staggers, temblique, loco, and huecù. From present reports the disease seems to be widespread, for it exists throughout the pampa at least as far north as the Chubut Valley and extends southward to Deseado and from the eastern boundary of the pampa to the Andes. The incidence varies greatly with the condition of the food supply; when there is a liberal amount of grass the actual number of cases is small. After a long continued drought when the fine grass supply is short, the number of sick animals is large. The mortality varies considerably, young sheep seeming to suffer most. The disease is not confined to the ovine species alone; horses and cattle succumb readily to it.

Acosta, in his publications on huecù,<sup>1</sup> discusses in detail the geographical distribution, symptoms, diagnosis, and prognosis of a disease similar to staggers.

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\* The field expenses of this investigation were borne by a sheep-farming company of London, which has not authorized the publication of its connection with the investigation, but which gave every facility for the work on its estancia in Patagonia. Here a small bunk house close to the settlement was converted into a temporary laboratory. Only the most necessary equipment and supplies were transported from New York.

<sup>1</sup> Acosta, J. L., Producción experimental de una enfermedad de tipo nervioso, *Rev. Zoot.*, 1914, v, 3; El "Huecù" o "Huaicù." Enfermedad de tipo nervioso propia de los herbívoros de la Patagonia, Tesis Universidad Nacional de Buenos Aires, No. 86, 1914.

His experimental feedings of a coarse tuft pampa grass (*Poa denudata*) to a horse, a bullock, and two sheep resulted in the production of definite nervous symptoms. Acosta's experiments were not controlled and inoculations were not attempted. He states that symptoms develop from 12 to 24 hours after the first feeding.

Conversation with a number of ranchers has given us some valuable data. The manager of an estancia in Chubut Territory supplied us with many interesting facts concerning staggers. On this particular farm about 1,000 sheep are pastured on a square league (9 square miles). The estancia consists largely of pampa<sup>2</sup> broken by many deep valleys. The water supply is excellent and consists of fresh springs coming from the hillsides close to the valley bottoms. There are many brackish water-holes as well. The herbage comprises several kinds of fine grass, two kinds of edible bush, and two coarse grasses, as well as several species of thorny bushes and a number of cacti.

Sheep were brought to this land in 1897. The land was at first unfenced and the sheep were herded. Lambs after weaning could be turned on virgin pasture. Even early in the history of the farm, employees would occasionally report a case of staggers, but it was only after the land had been fenced and animals permitted to feed over it for several years that the disease really became a menace. Beginning in 1910 more or less serious outbreaks of the disease have occurred annually. It affects lambs and hoggets (sheep under 1 year old), although adult sheep suffer to some extent. As a rule, the ewes lamb in August and the lambs are weaned in January and are then removed to separate paddocks. Early in February the malady may become epidemic and it may continue during the ensuing months until September. The incidence varies considerably with the general conditions of the pasture. When the seasons have been unusually dry and the herbage is sparse it is not unusual to see lambs affected at marking time.<sup>3</sup> The disease often exists at shearing in December. Under these conditions the nourishment has been insufficient since birth and the young have been forced to feed on grasses otherwise unnecessary.

<sup>2</sup> The term pampa in Patagonia is applied to the sandy plain beginning in the foot-hills of the Andes and sloping eastward toward the Atlantic Ocean. The vegetation of these plains is much sparser than that of those to the north.

<sup>3</sup> When lambs are 6 weeks old they are docked, marked, and castrated.

When there has been plenty of moisture and the grasses are sufficient, cases of staggers are not usually seen, either at lamb-marking or shearing, but a few cases may be observed in March and April. In extremely bad seasons the incidence may reach 100 per cent in certain paddocks. At this particular estancia the average mortality is about 5 per cent but in one paddock it reached 25 per cent.

In horses and cattle, especially in the wild herds, the losses from staggers are severe. Foals and calves from 4 to 9 months old are especially susceptible. In all paddocks located on the pampa the disease abounds. There are a few low lying paddocks, 200 or 300 feet lower than the pampa, where the disease has never existed. Sheep farmers whose lands occupy the low belt of land between the pampa and the sea declare that staggers is unknown in their flocks, although only a five strand wire fence may separate them from heavily infected pastures.

A sheep farmer whose estancia was located at Lago San Martin, about 250 leagues southwest of Camarones, informed us that his land would support 3,000 sheep to the league. Sheep had been grazed on the land for 7 years. During the first years staggers did not exist, but as the grasses became more sparse staggers became prevalent although not alarming. He stated that the young animals were affected shortly after weaning. The greatest number of cases occurred in the paddocks close to the settlement when large numbers of sheep were pastured temporarily during shearing and dipping. The fine grass in these paddocks had become very close. When questioned about the prevalence of a certain grass he informed us that it grew in all of his paddocks except those that had an elevation of over 1,500 feet. The average elevation of the paddocks was about 900 feet. Further questioning brought out the important point that he had never seen the grass commonly called pampa or coiron growing on the pampa below Deseado.

#### *Description of the Disease.*

At first sight the animal may appear normal. Excitement becomes great when the individual is alarmed by the barking of a dog, voices, etc. On becoming frightened it stands with a wild, excited look. The

neck is extended and there is usually a marked trembling of the head. Muscular twitchings of the hind legs are a constant symptom; if the animal is driven it usually breaks into a panic-stricken run. After running a short distance, stiffness of the limbs becomes marked. This stiffness is usually more noticeable in the hind legs. It moves with short, convulsive strides, and suddenly plunges forward, falling with the hind legs extended backward, and often rolls on its side (Figs. 1 to 4). If the sheep falls on a hillside it is not unusual for it to roll over and over until a level plain is reached. It is quite common to observe a sudden stiffness of all four legs; when this occurs the individual may fall directly on its side. When the animal falls it displays extreme excitability; the eyes bulge and the pupils are dilated. The head is drawn back, the muscles of the neck are tense, and the legs are extended rigidly from the body, with the digits spread far apart. If a sharp sound is made the muscles become more rigid. If the sheep is permitted to lie undisturbed the muscles gradually become flaccid, and the animal rises to its feet with some difficulty and moves away, stiffly at first, but the gait soon becomes normal. If it is frightened the same phenomena are repeated. Often there is an impairment of vision; animals may shy away from fences or attempt to jump over objects which are at a considerable distance.

When an animal has fallen and through weakness is unable to rise, it is usually found lying on its side (Fig. 5). At the slightest alarm it will make peculiar cantering movements of the legs until seized by the usual convulsions. It is not unusual to see a considerable excavation which has been made by the feet of such an animal. Often they are killed or mutilated by birds or carnivora.

Even in advanced cases of the disease, the temperature remains normal. The pulse is usually regular and weak. A ramiform congestion of the vessels of the conjunctiva is ordinarily present and the conjunctivitis is accompanied by a mucopurulent exudate. The sick animals eat well when the opportunity is afforded. There is no diarrhea or constipation.

#### *Morbid Anatomy.*

*Macroscopic.*—The following autopsy notes reveal the usual type of lesions found:

*Subject.*—Yearling hogget; wether; had been down at least 2 weeks. Pulse 68; temperature 101°F.; respirations 21. Blood: hemoglobin 90 per cent; red cells 10,608,000; leukocytes 11,500. Animal killed by chloroform; much emaciated.

*Heart.*—The pericardium appears normal. The heart muscle is apparently normal. The right auricle and the right ventricle contain large dark red blood clots. The left ventricle contains a smaller clot. All the valves are normal.

*Lungs.*—Right: On the surface of the middle lobe is a dark purplish red discoloration measuring 9 by 6 cm., rather clearly demarcated from the usual bright pink lung tissue. On section it is found to involve the pleura and invade the subpleural tissue for a distance of 0.1 cm. The rest of the lung is normal. Left: Scattered over the posterior lobe of the left lung are many irregular, raised, indistinct, grayish white areas varying in diameter from 0.1 to 2 cm. They are discrete and crepitate on pressure.

*Liver.*—The liver is dark red in color and somewhat firm. On section the color is dark red. The consistency appears normal. The gall bladder is filled with dark bile.

*Spleen.*—Apparently normal.

*Kidneys.*—Right: Normal in size and reddish gray in color when viewed through the capsule, which peels off readily. On section the consistency is normal. The cortex and medulla are congested. Left: The left kidney presents the same picture as the right.

*Pancreas.*—The pancreas is slightly congested but otherwise normal.

*Esophagus and Stomachs.*—All appear normal.

*Duodenum.*—The vessels of the duodenum are congested. The mucosa is congested. The content is largely composed of mucus. The ileum, jejunum, cecum, and colon appear normal.

*Brain.*—The vessels of the dura mater are slightly congested. The superficial vessels of the cerebrum, cerebellum, and medulla are all highly congested. Along the anterior border of the cerebrum and extending 1.5 cm. on both sides of the median fissure is a triangular smoke-colored discoloration which is superficial and extends backward, reaching an apex 2 cm. from the anterior border. On the ventral aspect of the cerebrum, almost exactly opposite the dorsal

area, is another irregular patch of the same color, which measures 2 by 1.3 cm.<sup>4</sup> On section the deeper vessels are congested.

The cerebrospinal fluid is pinkish gray in color. The spinal cord is slightly congested. The sciatic nerve appears normal.

In most animals autopsied the lesions were of about the same type. It is unusual to observe congestion of the duodenum. In a few individuals small hemorrhages had occurred about the superficial vessels of the brain. The cerebrospinal fluid varied from straw color to pinkish gray. In all cases there was a more or less marked congestion of the kidneys with a certain amount of granular degeneration of the cortex, and, in some instances, of the medulla.

The intestinal tract was always examined for worms. In only two instances were they found and then only in small numbers. Examination of the frontal and nasal sinuses usually revealed varying numbers of the larvæ of *Æstrus ovis*. These were found as frequently in normal sheep as in those suffering with staggers.

Small pieces of muscle from the fore and hind legs and from the diaphragm were examined with M'Gowan's method for the presence of *Sarcosporidia*.<sup>5</sup> They were present in only two samples and then in very slight numbers. Frozen section of these muscles failed to reveal the parasites to any great extent.

*Microscopic*.—Pieces of various organs from normal sheep and those suffering from spontaneous and experimental staggers were fixed in Zenker's fluid. The sections were stained with methylene blue and eosin. The changes noted are slight and not specific.

The cardiac and skeletal muscles contain *Sarcosporidia* in all classes of sheep examined.

The liver as a rule appears normal. The kidneys are usually pathologic. The lesions vary considerably from congestion to cloudy swelling. The cortex is usually the seat of the processes.

The brain is generally congested throughout. The meninges appear normal except for a submeningeal black pigment.<sup>4</sup> The nerve cells

<sup>4</sup> At first these discolorations were regarded as possible lesions of staggers, but they were found in normal brains as frequently as in the brains of sick animals.

<sup>5</sup> M'Gowan, J. P., Investigations into the disease of sheep called "Scrapie," with especial reference to its association with sarcosporidiosis, *Edinburgh and E. Scotland Agric. Rep.*, 1914.



fail to show degenerative changes when stained with methylene blue. Congestion of the spinal cord is usually noted. In several instances small hemorrhages were visible within the gray matter. Too much stress cannot be placed on them, however, as the animals were usually killed by bleeding from the jugular veins and carotid arteries.

The spleen, lymph glands, lungs and trachea, pancreas, and adrenals fail to reveal abnormalities.

*Bacteriological Findings.*—Although inoculations from the internal organs and the central nervous system were made into various media and incubated both aerobically and anaerobically, in the main the cultures remained sterile. In animals that had been down for indefinite periods it was not uncommon to find several species of cocci in the brain, spinal cord, and cerebrospinal fluid.

Great care was taken in obtaining portions of the brain for cultivation. The skin was dissected away and the skull flamed with a gasoline blow-torch. A large sterile trephine was used for drilling the skull. Various portions of the brain were removed through the trephined holes. The cerebrospinal fluid was drawn immediately into sterile pipettes. Usually 15 cm. of the spinal cord were removed for inoculation into various media.

Films from the internal organs, brain, and spinal cord were prepared and stained with various aniline dyes. Bacteria were not uniformly present in any of them.

#### EXPERIMENTAL.

In order to ascertain whether this disease was transmissible from one animal to another, a series of experiments was undertaken. Although indications in the field seemed to point to some other etiologic factor than microparasites, we undertook to establish this point definitely.

Our first series of five experiments consisted in attempts to transmit the disease by natural means and by inoculation.

*Experiments 1 to 5.*—Susceptible sheep were permitted to pasture with those suffering from staggers. The normal sheep remained so and those affected with the disease completely recovered.

Five yearling sheep and two young lambs were inoculated with normal salt

solution suspensions prepared from the central nervous system, viscera, and blood of sheep suffering from advanced staggers. The experimental animals were inoculated in various ways, some intraperitoneally, others intravenously, subdurally, and directly into the cerebral substance. The latter died promptly from shock. Chloroform anesthesia was resorted to when inoculations were made beneath the dura mater and into the brain substance. In a few of the inoculated individuals, considerable doses of the suspensions were introduced into the rumen with the aid of a stomach tube.

None of the inoculated animals developed suggestive symptoms. All remained healthy during our observation of over 2½ months. Adequate controls which were maintained throughout this series of experiments remained healthy.

Ten sheep from an outlying estancia on which staggers did not exist were purchased for experimental purposes. These animals were transported to the settlement and placed directly in disinfected pens. Some of these were used in the inoculation experiments and the others in the experimental feedings later.

Guinea pigs weighing 300 gm. were also inoculated with tissue suspensions from several cases of staggers. They remained well.

From the foregoing series of experiments it seemed well established that the disease could not be produced by permitting sick animals to come in contact with healthy ones. Moreover, in every instance when healthy susceptible sheep were inoculated with material obtained from advanced cases of staggers they failed to develop the disease.

Among certain individuals throughout the district, factors seemed to point to a grass as a possible cause for the disorder. The grass is commonly called coiron or pampa grass. Previously we have mentioned that staggers had not affected animals on low camps. The disease is confined to the pampa. It is well known that a coarse tuft grass grows in large quantities on the pampa at altitudes from 500 to 1,500 feet. The tufts may extend well down the sides of the valleys but they do not grow in the valley bottoms except close to the pampa where the canyons are shallow. It grows in tufts varying from 15 to 60 cm. in diameter. The height varies with the amount of moisture. Where the tufts have not been disturbed it may reach a height of 40 or 50 cm.<sup>6</sup>

<sup>6</sup> The writers wish to acknowledge their indebtedness to Dr. A. S. Hitchcock, Agrostologist of the Bureau of Plant Industry of the United States Department of Agriculture, who has identified this grass as *Poa argentina*.

A series of experiments was started to test the effect of this grass when fed to normal sheep and to those that had recently recovered from staggers.

*Experiment 6.*—Sept. 16, 1916. Four native yearling hoggets were placed in two small pens and fed on pampa grass. Four other animals of the same age and from the same flock were fed on alfalfa hay and served as controls. The water supply was the same in all the pens. The following notes were made during the experiment:

Sept. 20. Sheep 1 shows muscular trembling. Sept. 25. Sheep 1 is very excitable; marked weakness of hind legs; falls on becoming violently excited. Sept. 30. More violent symptoms exhibited by No. 1. Sheep 2 and 3 are highly excitable. Oct. 3. Sheep 1, 2, and 3 show characteristic symptoms of staggers; *i.e.*, short, jerky movements of the head from side to side. Sheep 1 on becoming excited loses use of the hind legs, crawls about with the fore legs, and drags the hind limbs (Fig. 6). Oct. 7. Sheep 4 shows slight shaking of the head, grinds the teeth, and has the characteristic stupid facial expression. Sheep 2 has become worse rapidly, showing the same symptoms as No. 1.

The notes of a complete physical examination of No. 1 were as follows (Fig. 6):

*Sheep 1.*—Half bred ewe; age 1 year. Muscular trembling, incoordination of the muscles of the hind legs, grinding of the jaws, constant defecation of hard feces during examination. The animal is slightly excited and restless. Temperature 104.8°F.; pulse 93, regular, full, and weak; respirations 116 (very warm day), rapid, shallow, and regular. The heart appears normal. The skin is loose and normal, the coat of wool excellent. The face is symmetrical with no evidence of inflammation of the lips, nostrils, or ears. There is a ramiform congestion of the conjunctiva accompanied with a mucopurulent exudate. The abdomen is distended. Micturition is frequent. Mental excitement is marked but the sensibility is normal. Muscular tremblings are constant and marked disturbances of the muscular sense are observed. The reflexes are normal.

The animal lies quietly during the examination. When assisted to rise and compelled to move, it trembles violently and appears weak. The hind legs are stiff and the fore legs are braced far apart.

Blood: hemoglobin 85 per cent; red cells 9,304,000; leukocytes 7,400.

Oct. 8. Animal 1 fell and was unable to rise. Oct. 10. Chloroformed.

*Autopsy.*—General condition good.

*Heart.*—The pericardium and heart appear normal.

*Lungs.*—Right, normal; left, contains one darkened area in the ventral lobe measuring 9 by 5 cm. It is clearly demarcated from the rest of the normal tissue. The lobe is somewhat congested.

*Spleen.*—Normal in size and consistency.

*Liver.*—Normal in size; the color is light brownish pink and the consistency firm. Superficially the intralobular markings are distinct. When cut the

liver appears firm and the lobules stand out clearly. The gall bladder is filled with bile.

*Kidneys*.—Both appear congested, otherwise normal. The bladder is empty. The suprarenals are normal.

*Pancreas*.—Apparently normal.

*Stomachs*.—All contain more or less partially digested grass; no evidence of inflammation.

*Intestines*.—The bowels appear normal until the jejunum is reached. Here the vessels are congested. The mucosa contains a few hemorrhagic areas varying in size from 0.2 to 0.4 cm.

*Ovaries*.—Apparently normal.

*Uterus*.—Contains a large well developed fetus.

*Brain*.—The meninges appear normal. The superficial vessels of the brain are engorged with blood. There is a grayish black discoloration on the anterior portion of both lobes of the cerebrum. This discoloration begins a distance of 2 cm. posterior to the anterior border and extends forward and downward to the ventral aspect. It is clearly demarcated from the rest of the tissue and lies only on the surface. The cerebellum and medulla appear normal. There is a normal amount of clear straw-colored cerebrospinal fluid. The superficial vessels of the spinal cord are congested.

*Bone Marrow*.—The bone marrow of the humerus and the femur appear normal. The marrow of the shaft is a dull pink color and of a stiff gelatinous consistency. That of the heads is spongy and bright red in color.

In certain tubes of the media inoculated with small pieces of brain, cocci appeared after incubation. All inoculations from the heart and liver remained sterile. Salt solution suspensions of various organs of this animal were used to inoculate the sheep employed in Experiment 5.

The other animals in the experiment gradually became worse, all developing symptoms of advanced staggers, finally falling down and becoming unable to rise.

Oct. 25. Sheep 3 fell down. Nov. 7. Sheep 2 and 4 unable to rise.

The autopsies of these animals failed to show any more characteristic lesions than those found in Sheep 1. Although careful examinations of the intestines and stomachs were made, intestinal worms were not found. A common occurrence among the sheep autopsied was the presence of the larvæ of *Æstrus ovis* in the frontal sinuses.

In all these animals examinations of the muscles with M'Gowan's method for the presence of Sarcosporidia were made. In one or two instances they were present, but in very small numbers.

Unfortunately the preceding experiment was carried out with native stock. Doubtless these animals had been exposed to the disease. The results seemed to justify a repetition of the experiment with the substitution of animals which had never been exposed to staggers.

*Experiment 7.*—Oct. 5, 1916. The sheep used were of the same lot as those described under the inoculation experiments. Adequate controls were kept.

Within 3 days after starting to feed the grass one animal exhibited suspicious symptoms. 2 days later two animals revealed characteristic symptoms of staggers. The other two sheep developed staggers 10 and 15 days respectively after the feeding had been begun.

One animal (No. 5) after showing distinct symptoms of staggers was unable to maintain itself on the grass and was therefore killed. The others became progressively worse, falling down at the slightest excitement (Figs. 7 and 8).

The following results were noted when the urine of the three surviving animals was examined.

*Sheep 6.*—Amount, 10 cc.; reaction alkaline; color golden yellow, turbid; albumin present; sugar, bile, blood, and casts absent.

*Sheep 7.*—Amount 8 cc.; reaction alkaline; color golden yellow, turbid; albumin present; sugar, bile, blood, and casts absent.

*Sheep 8.*—Amount 8.5 cc.; reaction alkaline; color golden yellow, clear; albumin present; sugar, bile, blood; and casts absent.

All the animals were finally autopsied but failed to reveal more characteristic changes than those recorded.

In Experiments 6 and 7 the pulse, respiratory rate, and temperature of each animal were recorded before and during the experiments. The temperatures varied slightly and the rate of the pulse and respiration remained practically the same.

*Experiment 8.*—Nov. 7, 1916. In Experiments 6 and 7 the grass used was gathered from a paddock and stored in large bags until ready to feed. The grass tufts were cut close to the bottom and the whole tussock was used. A large proportion of the tufts consist of a dry, dead center surrounded by a more or less profuse belt of green freshly growing grass. Although we had never seen sheep eat this dry center, nevertheless it was believed by many that the dry portions were responsible for the disorder. To prove definitely whether this was responsible for the malady we determined to feed only green grass from young tussocks. In the young tufts the whole mass is green and only begins to dry as the seed ripens. Every morning 5 kilos of this fresh green grass were gathered and fed to two sheep of the same lot as those used in Experiment 7. Two animals fed on alfalfa in an adjoining pen served as controls.

After 2 days' feeding on the grass both animals showed suspicious symptoms; *i.e.*, twitching of the muscles of the hind limbs, slight shaking of the head, and twitching of the ears. At the end of 1 week's feeding they had developed severe symptoms of staggers (Fig. 9). 14 days after feeding had been begun both animals fell on the slightest excitement.

Examination of the urine gave the following results:

*Sheep 9.*—Amount 45 cc.; reaction acid; color golden yellow, clear; specific gravity 1,060; albumin present; sugar, blood, bile, and casts absent.

*Sheep 10.*—Amount 150 cc.; reaction acid; color light golden yellow; specific gravity 1,021; albumin and sugar present; blood, bile, and casts absent.

Sheep 9 fell on the 15th day of feeding and could not rise. It was killed and autopsied on Nov. 22. Unusual changes were not noted.

The other animal, although badly affected with staggers, was able to stand, and after two feedings of green alfalfa it was turned out with two other sick animals (see Experiment 11).

*Experiment 9.*—Feeding of pampa grass to sheep that had recently recovered from staggers. Sept. 30, 1916. The feeding of pampa grass was begun with two sheep that had completely, and one that had partially recovered from staggers. These animals were part of a flock in which the disease had existed. They, with nine others, had been isolated in a small paddock about 2 weeks before our arrival and were to have furnished material for our investigation. Of these twelve animals, nine had recovered, two had become worse, and one had partially recovered. The partially recovered animal still showed extreme excitability and muscular trembling.

Oct. 5. One of the recovered sheep revealed unmistakable symptoms of staggers. The partially recovered animal had become much worse and fell on becoming excited. 2 days later the other hogget developed staggers. These animals all became worse and were either autopsied or used for other experiments.

When the three preceding experiments are reviewed it will be noted that of ten yearling sheep whose diet consisted of pampa grass all developed staggers. The controls fed on alfalfa hay remained well. Another striking feature is that both animals fed on fresh green pampa grass showed symptoms of the malady as rapidly as any of the others. The latter observation eliminates the theory of an infestation of the grass by a toxin-producing mold. The green grass we fed was free from mold.

It has been stated that old animals do not usually suffer from staggers unless they have been reared on low land and moved to the pampa. In instances of this kind, the losses may be severe. In the main, older animals that have been pastured on pampa cannot be said to suffer from the malady to any great extent. We are informed that the guanaco, a native member of the deer family, does not suffer from staggers. Indications on all upland paddocks visited showed that considerable coiron is eaten by animals. Often the green blades were kept well cropped. Knowing that more or less of the grass is eaten by older animals and that comparatively few cases of

staggers make their appearance in these flocks, we assumed that a tolerance to the toxic properties of the grass must have been developed. To test this point it was determined to feed the grass to older animals.

*Experiment 10.*—Nov. 14, 1916. In this experiment it was decided to feed adult animals on pampa grass. A 6 year old ewe with a 3 months old lamb, and two 2 year old wethers were fed upon pampa grass. The ewe could not support herself and the lamb on the diet and was discarded. The lamb showed symptoms of staggers within 8 days.

After 23 days of feeding on the grass, neither wether had developed staggers. Since it was necessary to return to New York, we requested the manager to continue feeding the grass and to telegraph us when the symptoms developed.

On Dec. 28, 45 days after the beginning of the feeding, he telegraphed us that both animals had staggers.

When young animals were fed on the grass, the average time for the first appearance of definite symptoms of the disease was 10 days. At the end of 23 days neither of the older animals revealed suspicious symptoms; in fact, 45 days of actual feeding were required to produce the disease. It seems that a considerable tolerance is developed in adult animals that have pastured on land on which the grass grows. The results of this experiment readily explain why these sheep do not suffer from the disorder to any great extent. In all probability the guanaco, through generations, has developed an immunity against the toxic substances of the grass.

*Experiment 11.*—In spontaneous outbreaks of the disorder the mortality is usually slight when compared with the incidence. In fact, in certain outbreaks all the animals recover if there is sufficient green fodder.

Sept. 16, 1916. Three yearling rams were brought into the settlement by the manager and placed in a small corral; these animals presented all the symptoms of staggers and would fall on becoming frightened (Fig. 10). They were of value for breeding purposes and the manager determined to treat them. Sept. 16 and 17. Each was given a pint of oatmeal gruel and permitted to eat rolled oats and both dry and green alfalfa. The bulk of the diet consisted of alfalfa hay. Within 1 week the improvement was marked; even when they were driven by dogs they did not fall. The symptoms rapidly disappeared and within 18 days all had recovered (Fig. 11).

In another instance, two animals, the lamb from Experiment 10 and a hogget from Experiment 8, together with a spontaneous case, were allowed to run in a small paddock where the finer grasses were plentiful. One of the animals could not be driven but had to be carried to the paddock; at the least excitement it

would fall and only rise with difficulty. Within 8 days all symptoms had disappeared and all three sheep had completely recovered.

*Experiment 12.*—Attempts were made to extract some substances from the grass that would cause the symptoms. Acosta was unable to state definitely whether the plant itself contained an alkaloid or whether a toxin was produced by the action of a mold. The former seemed more plausible as Patagonia is an exceedingly dry country. Moreover, the experiment in which we fed green grass which was not moldy seems to point to an alkaloid.

At first we attempted to extract a substance from the plant with alcohol, but on account of our small supply of that chemical we were compelled to discontinue this portion of the experiment.

We next attempted to extract some substance aqueously. This was done in the following manner: 3 kilos of grass were washed twice and then covered with water and allowed to boil for 2 hours. The liquid was then poured off and used to cover a second 3 kilos of washed grass. This was repeated a third time. The dark brown liquid thus obtained was evaporated to 1 liter. It was necessary to prepare a large quantity 3 or 4 days before the administration of the liquid was begun, to enable us to continue the supply.

Two yearling hoggets were chosen for the experiment. The fluid was administered directly into the rumen by means of a stomach tube. Each animal received 1 liter before feeding in the morning and another liter in the evening, so that each animal was receiving daily the infusion from 18 kilos of grass. The experiment was continued for a period of 33 days, but symptoms failed to develop in either of the sheep.

Guinea pigs, when injected intraperitoneally with heavy alcoholic or aqueous extracts of the plant, usually died within 15 minutes. Controls inoculated with similar preparations from non-toxic grasses died as promptly. It was impossible to attempt to purify the products and it seemed that these results may be accounted for by an excess of salts in the fluids.

A young hogget was injected intraperitoneally with a large dose of a heavy grass infusion. It developed peritonitis and died within 12 hours. Symptoms of staggers were not observed.

It seemed that animals affected with staggers or those that had recovered might react when a few drops of liquid thought to contain the toxic substance were placed in the eye. Both alcoholic and aqueous extracts were evaporated to dryness and the residue was taken up in distilled water. Normal, affected, and recovered animals received two drops of these solutions in each eye. All failed to react.

Probably only more refined and exhaustive chemical methods will be able to decide the nature of the substance which is responsible for the symptoms.



## DISCUSSION.

There seems to be very little doubt that staggers in sheep in Patagonia is caused by ingestion of a coarse tuft grass. This plant is called pampa or coiron grass. Its botanical name is *Poa argentina* (Fig. 12). All young sheep (yearlings) when fed on this grass developed characteristic symptoms of the disease. Besides our experimental evidence, there exists a number of other facts which corroborate our findings. Certain of these can best be illustrated by a map of an estancia on which staggers is prevalent (Text-fig. 1).

The larger paddocks consist of broken pampa; *i.e.*, pampa intersected by valleys. In all of them except the Windmill Paddock, the water supply is natural. The windmill wells have been driven. In all the upland paddocks staggers exists. The pampa grass grows well. On this property there are three low paddocks (Nos. 1, 2, and X) where this particular grass does not grow. These paddocks are at least 200 or 300 feet lower in altitude than the pampa. No matter how sparse the herbage may become in them staggers never develops in any of the sheep ranged there. This company owns a piece of land six leagues to the eastward and only a few feet above sea level; on this tract the disease does not exist and the coiron grass is not found. When a flock of 2 year old ewes was moved from these low camps to the mixed pampa paddocks, staggers broke out.

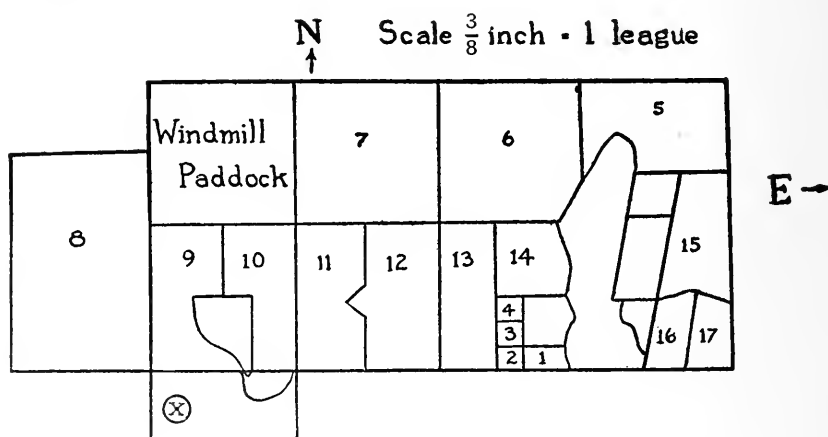
The question may be raised whether the symptoms may be caused by a lack of some vital substance in the diet; *i.e.*, whether staggers is an insufficiency disease. There are several facts which lead us to believe that the disorder is not of such a nature. When horses are brought on the pampa from other districts they often come down with staggers within 24 hours. We have seen sheep reveal symptoms after two feedings of pampa grass. In years of drought many animals die of starvation in the low camps where the pampa grass does not grow but the other food is the same. These sheep do not develop nervous manifestations.

Theiler, Green, and Viljoen<sup>7</sup> have shown that even after long periods

<sup>7</sup> Theiler, A., Green, H. H., and Viljoen, P. R., Contribution to the study of deficiency disease, with special reference to the Lamkziekte problem in South Africa, *Third and Fourth Reports, Union of South Africa, Dept. Agric.*, 1915.

horses, cattle, and sheep fed on a diet of ten parts of polished rice and one part of autoclaved hay failed to develop symptoms of an avitaminosis.

It would seem that the solution of the problem lies in the eradication of this particular grass (Fig. 13). When the fact is considered that this grass is abundant from the foot-hills of the Andes to the eastern boundary of the pampa, one gets an idea of the magnitude of the problem. During the summer months when the grass is seeding, the prevailing winds are from the west and it is not unusual for them to blow steadily for 3 or 4 days. At one time during our stay the



Paddocks 3 to 17 and the Windmill Paddock on pampa land.  
Paddocks 1 to 2 and ⊗ on low land.

TEXT-FIG. 1. Map of an estancia on which staggers is prevalent.

wind reached a maximum velocity of 84 miles an hour. Even though this grass is eradicated from eastern portions of the land it would soon reappear. One land owner tried burning off a portion of land but this failed to kill the coiron. Unless another grass can be substituted, the eradication of this particular species might work considerable havoc. There is no doubt that old sheep that have become tolerant eat it without harmful effects. Probably during periods of drought many animals are dependent on it for certain nourishment. Without the substitution of some other bulky food the losses from

starvation might well equal the losses occurring from staggers in continued periods of drought.

Individual treatment is valueless on large estancias at the present time. Many of the valleys in this district are admirably suited to the raising of alfalfa after proper treatment of the soil. Generally two good cuttings may be obtained annually after a few years. In the future when the value of sheep has advanced it may pay to cultivate portions of the better valleys and either use the alfalfa as a green forage or store it as hay or ensilage and feed it in times of stress. It is recognized at this time that this procedure is impracticable, although it may become necessary in the future.

Sheep raisers have long recognized the advisability of not exciting flocks in which many cases of staggers exist. Usually when flocks in which the incidence is heavy are driven by men and dogs, the sheep, becoming violently excited, attempt to run and many of them fall down. Of those that fall many are unable to rise and ultimately die of starvation or are killed or mutilated by animals or birds. Quiet is essential when many are sick. On the other hand, it may be advisable to move sheep to another pasture early in the outbreak if this is possible.

Acosta recommends the use of nose bags when sheep are driven through lands on which the grass exists. When it is considered that two or three men with dogs usually drive bands of a thousand or more sheep, such a procedure seems impossible.

Staggers affects horses and cattle as well as sheep. Acosta was able to produce the disease experimentally in both species by feeding *Poa denudata*. Apparently *Poa denudata* and *Poa argentina* differ in but one or two minor characteristics. Opportunity for experiments along this line was not afforded to us.

#### CONCLUSIONS.

After observations and experimental work both in the field and laboratory, the following conclusions seem justified.

1. Staggers is a non-infectious disorder affecting horses, cattle, and sheep.

2. The disease is characterized by weakness, muscular twitching, irregular movements of the head, stiffness of the limbs, and transient

motor paralysis, accompanied with spastic spasms on excitement. There is also a derangement of vision and conjunctivitis.

3. The postmortem lesions are not characteristic.

4. We readily produced the disease by feeding susceptible sheep on a coarse tuft grass commonly known as coiron or pampa grass (*Poa argentina*).

5. The time required to produce definite symptoms by feeding the grass varied. Two animals developed typical staggers after two feedings; in another instance a period of 21 days of feeding was required. The average time for the production of unmistakable symptoms in our experiments was 10 days.

6. Many sheep recover from staggers spontaneously. A complete change of diet will usually effect a cure within 2 weeks.

7. Older animals that have pastured for long periods on lands where the grass grows become tolerant and are rarely affected with staggers.

8. The grass is toxic to sheep at all seasons of the year. We fed late winter and early spring grass and grass in flower, and produced staggers in every instance. The young green grass is as toxic as any edible portion of the plant.

#### EXPLANATION OF PLATES.

##### PLATE 62.

FIGS. 1 to 5. Spontaneous staggers.

FIG. 1. Animal about to fall on its side; the fore legs are rigidly extended and the digits spread far apart.

FIG. 2. The transient motor paralysis of the hind limbs occurring after violent exercise and excitement.

FIG. 3. Sheep about to fall. All four legs are extended rigidly. The momentum gained was sufficient to carry the body forward. The animal appears to plunge forward while falling.

FIG. 4. The gait of sheep with staggers; note the apparent rigidity of the hind limbs.

FIG. 5. The usual position of an animal down with staggers.

##### PLATE 63.

FIGS. 6, 7, and 8. Experimental staggers.

FIG. 6. Sheep 1, Experiment 6. Note the stiff carriage of the hind limbs and the depression of the hind quarters.

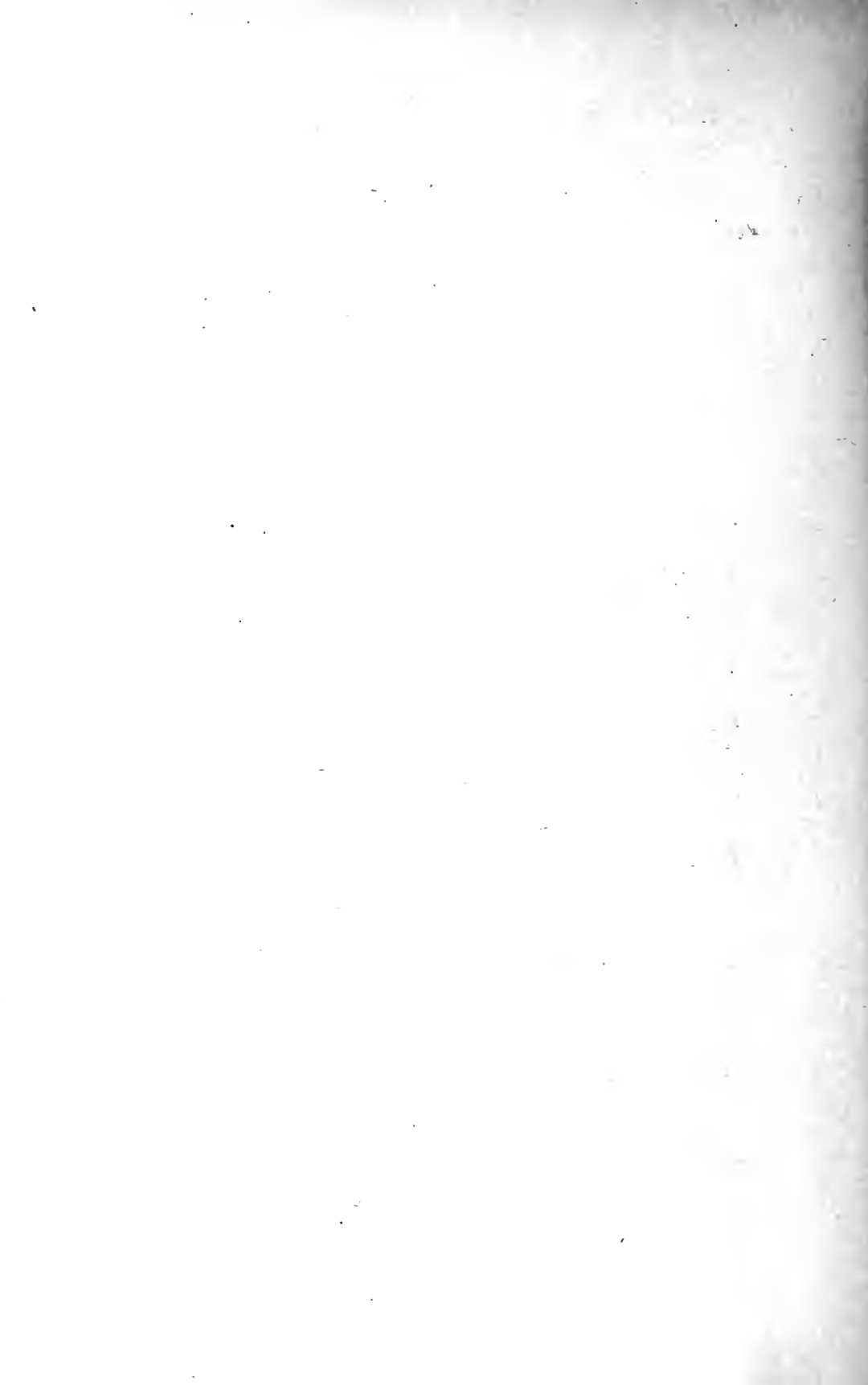


(Jones and Arnold: Staggers in sheep.)





(Jones and Arnold: Staggers in sheep.)

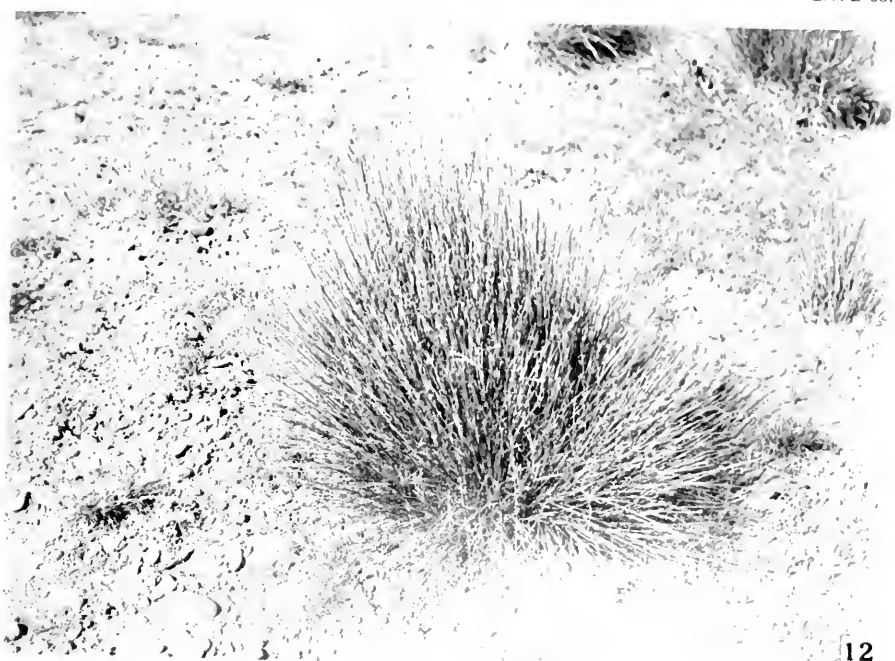






(Jones and Arnold: Staggers in sheep.)





12



13

(Jones and Arnold; Staggers in sheep.)



FIG. 7. Sheep 7 and 8, Experiment 7. Typical standing position of sheep affected with staggers.

FIG. 8. Sheep 8, Experiment 7. This plate was exposed  $\frac{1}{4}$  second and shows the characteristic shaking of the head.

PLATE 64.

FIG. 9. The sheep fed on green pampa grass (*Poa argentina*) in Experiment 8.

FIG. 10. Young rams affected with staggers (Experiment 11).

FIG. 11. The same animals as in Fig. 10, completely recovered after feeding for 18 days on a mixed diet of alfalfa and rolled oats.

PLATE 65.

FIG. 12. Pampa grass (*Poa argentina*) in flower.

FIG. 13. The grass growing on the pampa close to the edge of a valley.



# THE DIVERSION OF THE PANCREATIC JUICE FROM THE DUODENUM INTO THE STOMACH. ITS EFFECTS UPON THE LEVEL OF GASTRIC ACIDITY AND UPON THE PANCREAS.

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Recent work has shown the important part played by the alkaline duodenal contents, the pancreatic juice in particular, in the maintenance of a uniform level of gastric acidity. While the presence of bile and pancreatic juice in the stomach was noted a number of years ago by Boas, it remained for Boldyreff (1907-08, 1911) to demonstrate experimentally that a regurgitation of duodenal juices, after the ingestion of certain foodstuffs, is of such regular occurrence that the process may be regarded as an accompaniment of normal gastric digestion. Confirmatory evidence for the existence of the mechanism responsible for this regurgitation has since been offered by Carlson (1915, *b*) Hicks and Visser, Zaitseff, Spencer, Meyer, Rehfuess, and Hawk, Morse, and others.

It has been demonstrated, furthermore, that neutral, alkaline, or feebly acid fluids provoke a secretion of acid juice in order that the gastric acidity may be brought to the normal level. The experiments of Lönnqvist, for example, have shown that when weak soda solutions (0.25 to 0.5 per cent) are introduced into the stomach they act like water in causing a secretion of gastric juice. With stronger concentrations (1.0 to 1.5 per cent) the amount of secretion is materially increased. While it is not inconceivable that strong alkaline solutions, acting as an irritant to the duodenal mucosa, may lead, indirectly, to an out-pouring of acid in the stomach, it is not likely that the weaker solutions act upon the gastric glands in this way. In fact Lönnqvist found that when solutions of soda were placed in the duodenum they led to a slightly diminished secretion of gastric juice.

There is but little evidence, however, to show how the level of gastric acidity is affected when alkaline solutions are introduced into the stomach, more or less continuously throughout the period of digestion. In view of the findings of Boldyreff (1911) which indicate that the gastric contents become more acid (hyperacidity) when there is an insufficient regurgitation of duodenal juices, one might expect to find a lowered level of acidity in the presence of an excessive duodenal regurgitation. When fats are fed in considerable amounts they bring about

a profuse influx of duodenal contents into the stomach, and at these times there is a decrease in the acidity level. But, as fat is only slightly affected by gastric juice, these experiments merely serve to demonstrate how the duodenum may aid the stomach in its struggle with an unusual burden.

While it would be difficult, experimentally, to increase or prolong the duodenal regurgitation without introducing fat or strongly acid solutions into the stomach, evidence bearing on this problem may be obtained by preparing animals in such a way that an unusual amount of bile or pancreatic juice enters the stomach throughout the period of digestion. In an earlier report (Grey) experiments were cited in which the total output of bile was made to flow through the stomach in its passage to the intestines. The present communication concerns certain additional experiments in which the pancreatic juice was diverted from the duodenum into the stomach.

The results of the first investigation appeared to indicate that the presence in the stomach throughout the course of digestion of the entire output of bile does not materially affect the level of gastric acidity. While no interpretation of these findings was attempted in the published report of the experiments, it was apparent, nevertheless, that they did not necessarily imply that bile is a feeble, neutralizing agent, but suggested, rather, that there had been a compensatory increase in the secretion of hydrochloric acid whereby an approximately normal level of acidity was maintained.

The analysis of the pure juice obtained from the smaller (Pawlow) stomachs did not reveal any appreciable changes in the hydrochloric acid content of the secretion secured subsequent to the introduction of bile. This observation was in accord with the findings of Carlson (1915, *b*) which point to a constant and uniform acidity of the digestion juice when it is secreted at a fairly high rate. A uniformly lower acidity is found under other circumstances; *i.e.*, in the empty stomach and in the continuous or hunger secretion, when the secretion rate is low.

The protocols of these experiments, however, indicate that there was a definite increase in the quantity of the digestive juice secreted after the bile had been diverted into the stomach. This conclusion has been based upon a comparison of the average of between twenty-five and thirty measurements made previous to the anastomosis with a similar average of a like number of measurements carried out subsequent to the operation. Inasmuch as there is some variation both in the quantity and in the acidity of the specimens secured from day to day under approximately constant conditions, it is not feasible to compare the data from any two single observations. The figures taken from the protocol of Dog 2 and published in Table I of the report under discussion were chosen because they furnished an accurate picture of the average acidity values, before and after the anastomoses. They indicate, however, differences only of about  $7\frac{1}{2}$  per cent in the two outputs. In this respect the table was misleading since a comparison of the average amounts of juice secreted before and after the anastomoses revealed a considerably greater difference than this.



It was obviously impossible to make quantitative studies of the hydrochloric acid output in the animals in which samples of the gastric contents were analyzed—apomorphine experiments. But since no material change in the acidity level was noted after the introduction of the bile, it seems justifiable to infer that a compensatory increase in the gastric secretion had ensued to counterbalance the unusual quantity of alkaline fluid in the stomach.

Analyses of the secretions of the upper intestinal tract have shown that a number of them are capable of neutralizing more or less acid in the gastric juice; *i.e.*, saliva, gastric mucus, pyloric juice, the secretion of Brunner's glands, succus entericus, bile, and the pancreatic juice. Of these the pancreatic juice is by far the most important, since the alkalinity of this secretion is half again as great as that of all the other secretions combined (Boldyreff, 1914-15).

Because of the marked alkalinity of the pancreatic juice and in view of the results obtained from the bile experiments, it seemed important to study the effects on the level of gastric acidity of an increased supply of pancreatic juice in the stomach. In the experiments to be cited the larger pancreatic duct of each animal was transplanted into the wall of the stomach and the lesser duct ligated and divided.

This procedure afforded an opportunity to make certain additional observations. It has been the view of some workers that the hydrochloric acid in the duodenum is responsible for certain of the pathological changes which are found in the pancreas. Hlava, for example, suggested that by antiperistalsis hyperacid gastric juice may be forced from the intestine into the pancreatic duct, thus causing hemorrhagic pancreatitis. If this postulation is valid in any sense, then an experimental procedure such as that mentioned should afford a favorable opportunity for the gastric juice to reach the gland and set up acute inflammatory or chronic sclerotic changes.

Flexner injected hydrochloric acid solutions ranging in strength from 0.5 to 2 per cent into the pancreas and through its duct and subsequently observed profound changes in the organ corresponding in degree to the amount of acid used. The pathological picture was that of hemorrhagic pancreatitis. However, though these studies demonstrated that hydrochloric acid, as well as certain other agents, is capable of producing an acute inflammatory degeneration, Flexner did not offer his results as evidence confirming the hypothesis of Hlava.

Dr. Halsted's instructive case of stone in the ampulla of Vater and Opie's experiments have shown the great significance of one of the duodenal juices—bile—in pancreatic diseases. But the manner of entry of the bile into the pancreatic duct in these instances is essentially different from that suggested by Hlava. In Opie's opinion there is no satisfactory proof to indicate that the hydrochloric acid is instrumental in bringing about such a disease.

### *Methods.*

The present report is based upon the results obtained from experiments carried out on seven dogs. A number of additional animals were used in the early part of the work, but they were employed solely in solving the surgical aspects of the problem.

Inasmuch as the principal purpose of the investigation was to follow the changes in the acidity level of the stomach, it seemed best to analyze specimens of the test meals instead of studying the pure gastric juice as was done in some of the bile experiments referred to above. For this purpose the gastric fistula as devised by Janeway proved to be satisfactory. All the animals were anesthetized with ether.

A small three-sided piece of stomach wall was turned down toward the greater curvature. This constituted a rectangular flap with its blood supply intact. By sewing the lateral margins together and closing the opening in the gastric wall a small tube was formed which was lined throughout by mucous membrane. The distal end was then sewed into the abdominal wall, care being taken to anchor it to the peritoneum, fascia, and skin. The mucosa and skin, of course, were sutured together. In experimenting with the reconstruction a very important feature was noted. During the first weeks subsequent to the operation there is a marked retraction of the new tube toward the peritoneal cavity, with a definite contraction of the external opening. The effects of these changes, however, may be greatly minimized if care is exercised during the operative procedure to anchor the tube in the abdominal parietes so that it protrudes for several centimeters above the level of the skin. This may be done without embarrassing the blood supply. It leaves a wide cuff of gastric mucosa exposed on the surface. When the skin margins are loosely attached to the latter, a considerable contraction of the opening may

take place without in any way stenosing the canal. Fistulas constructed on this plan will remain patent for many months. Frequent catheterization with only the most gentle manipulations serves to keep the tract clean.

Inspection of one of these fistulas 3 or 4 months subsequent to its construction shows a lead-pencil-like tube, 4 or 5 cm. in length, extending through the peritoneal cavity from the abdominal wall to the stomach. This arrangement probably interferes but little with the normal gastric movements. After the heartiest meal and even during emesis there is no loss of stomach contents from the canal.

While the experiments are still in progress concerning the transplantation of the pancreatic duct, devoid of duodenum, into the stomach wall, no satisfactory results of this nature were secured during the course of the work reported here. However, it was found possible to resect a very short cylinder of duodenum, approximately 2 cm. in length, containing the orifice of the major pancreatic duct, and, after closing one end, to transplant the opposite opening into the posterior wall of the stomach. Since this segment of small bowel devoid of its blood supply may atrophy and lead, as one of the earliest specimens demonstrated, to a stenosis of the duct, it was found necessary always to preserve the vessels of the transplanted duodenum. When care is exercised this may be accomplished without great difficulty. An end to end anastomosis then established the continuity of the duodenum. The lesser pancreatic duct which opens into the duodenum together with the common bile duct was now identified by dissection, doubly ligated, and divided.

While the transplantation of the pancreatic duct leads to a displacement of the adjoining parts of the pancreas, the new arrangement does not cause undue tension on the vessels which supply this organ. The operation is readily carried out through a high right rectus or midline incision.

A standard mixture of 70 gm. of ground, raw, lean beef and 75 cc. of tap water was used as a test meal. Samples were withdrawn for analysis 2 and 3 hours after the ingestion of the meal. These periods were chosen for two reasons. In the first place, it was found that at shorter intervals following the ingestion of the test meals the food had not undergone sufficient digestion; and after longer intervals

it was frequently very difficult to secure specimens for analysis, the ingesta apparently having left the stomach in large part. In the second place, it has been shown (Carlson, 1915, *a*) that the quantity of gastric juice yielded by a dog's accessory stomach (Pawlow) during the first 2 hours on a moderate meal of meat is about one-half that secreted for the entire digestive period. And from McClendon's experiments on man it appears that the acidity of the adult stomach rises during the first  $1\frac{1}{2}$  to 3 hours subsequent to a meal, after which it remains stationary until the food has nearly all left the stomach.

Care was taken to conduct the observations in the forenoon 18 hours after the last meal. The dogs received the same diet each day. Water was withdrawn after the test meals had been given. But throughout the balance of the day the animals were permitted to take as much water as they desired. The amount of exercise, the quantity of food, and the environmental conditions remained constant throughout the weeks of study.

Several dozen test meals were given in each case. The average results obtained under these conditions were taken to represent fairly accurately the normal acidity of the chyme. In withdrawing samples for analyses care was exercised to discard the contents of the fistula itself. The end of a soft rubber catheter gently introduced a few centimeters into the stomach cavity was connected with a stoppered flask. Attached to one arm of the latter was a large syringe. The suction created by withdrawing the plunger usually resulted in a discharge of sufficient juice for study.

For the quantitative estimation of trypsin in the gastric contents Spencer's modification of the Ehrenreich test was used.

The acidity determinations were made by titrating 1 cc. of the sample against  $\frac{N}{40}$  sodium hydroxide, using dimethylaminoazobenzene and phenolphthalein as indicators. The values have been expressed as the number of cubic centimeters of 0.1 N sodium hydroxide necessary to neutralize 100 cc. of gastric contents.

#### RESULTS.

Early in the course of the work it was noted that when test meals were given to the same animal on different days, under conditions

as similar as possible, samples of the gastric contents showed considerable variation in the hydrochloric acid content. Corresponding observations had been made during the course of the bile experiments discussed above. It was necessary, accordingly, to repeat the analyses a great number of times and average the results in order to secure an accurate picture of the acidity level at the periods of digestion chosen; *i.e.*, 2 and 3 hours after the ingestion of the meal. This mean was established both before and after the transplantation of the duct for each animal.

TABLE I.

Dog No.	Before transplantation of the duct.		After transplantation of the duct.		Interval between the fistula opera- tion and the transplan- tation.	Interval between the transplantation and the first analysis.
	2 hrs.	3 hrs.	2 hrs.	3 hrs.		
1*	104†	93	113	67	days	days
2*	107	100	122	74	30	33
3*	108	122	125	98	32	22
4*	103	115	112	83	45	28
5*	91	79	85	57	47	29
6*	96	82	91	67	54	15
7‡	112	120	108	73	55	20
					107	74

\* Test meal: 70 gm. of meat plus 75 cc. of water.

† Acidity values are expressed as the number of cubic centimeters of 0.1 N sodium hydroxide necessary to neutralize 100 cc. of gastric contents.

‡ Test meal: 100 gm. of meat plus 70 cc. of water.

In Table I the acidity values for each dog represent the average of the figures obtained in from ten to thirty separate analyses. The necessity for this mean has been emphasized by Foster and Lambert who believe that limitations of variability must be computed for each animal experimented upon, and that the only idea of limitation of function that we can gain must depend upon an average computed from the data for a number of animals. In the paper of Long and Hull the tables indicate a similar variation of the acid content from day to day.

In order to ascertain whether any relation might exist between the level of gastric acidity and the interval of time elapsing from the date

of the operative procedure to that of the analyses, the time factor was changed throughout the experiments. Though the animals were studied over varying periods, from 100 (Dog 5) to 228 days (Dog 7), no such relation was discernible.

From a study of the figures in the table it is evident that in three animals (Dogs 5, 6, and 7) the acidity level of the gastric contents was lowered by the introduction of pancreatic juice into the stomach, both at the 2 hour and at the 3 hour period of digestion. In Dog 7 the reduction was especially marked during the second period. On the other hand, the analyses in four of the animals (Nos. 1, 2, 3, and 4) showed a slight increase of acid in the 2 hour specimen, but a decrease in the specimens secured at the 3 hour period. While four of the seven dogs were thus found to have a higher level of gastric acidity subsequent to the transplantation of the pancreatic duct at the 2 hour period, it is noteworthy that in all the animals the acidity level fell at the 3 hour period. Moreover, in each of the latter, following the transplantation of the duct, the percentage of hydrochloric acid in the stomach contents was less at the 3 hour period than it was previous to the operation at the 2 hour period.

A further analysis of the table shows that in three dogs (Nos. 3, 4, and 7) before the pancreas had been disturbed, specimens of test meal contained more acid 3 hours after the ingestion of the food than an hour earlier than this; whereas the relationship was reversed in the remaining animals. These findings probably have their explanation in the fact that the dogs varied in size. In the smaller animals the process of gastric digestion was more prolonged. Subsequent to the operations, however, the specimen secured in the second period of digestion, in every case, contained less acid than that withdrawn during the first period. The constancy of this finding we may fairly attribute to the altered chemical conditions in the stomach induced by the pancreatic juice.

The experiments as a whole thus appear to indicate that the presence of a large quantity of pancreatic juice in the stomach throughout the period of digestion leads to a moderate reduction of the acidity level in the later stages of digestion. Earlier in the process there is no constant alteration of the acid content in either direction. The surprising feature of these results would seem to be the maintenance

of a relatively high acidity level in the presence of the large amount of pancreatic juice.

In view of the part played by the pancreatic juice in the self-regulating mechanism described by Boldyreff one might have anticipated, under these circumstances, a material reduction in the acidity level. The absence of such a result, however, is really in keeping with Boldyreff's hypothesis.

One of the controlling factors in the mechanism is the mucosa of the duodenum. Hyperacid stomach contents reaching this part of the digestive tract irritate the mucous membrane, and the latter, in turn, serves to bring about a reduction of the acidity level of the stomach through a regurgitation of alkaline duodenal juices. This type of self regulation, however, ensues only when the hydrochloric acid content of the chyme is above normal. When alkaline materials have lowered the acidity level a different process is called into action.

Experiments have shown that following the introduction of alkaline solutions into the stomach the gastric glands respond with a discharge of juice in an endeavor, it would appear, to restore the gastric acidity to the normal level. The presence of a considerable quantity of pancreatic juice in the stomach, of course, leads to a similar disturbance of the acidity level. Accordingly under these circumstances one would also expect to find an increase in the gastric secretion. Now it has been shown (Table I) that a more or less continuous influx of pancreatic juice into the stomach throughout the period of gastric digestion only depresses the acidity level in the later stages of digestion. This we can explain only by assuming that in the experiments mentioned there was an augmented secretion of hydrochloric acid throughout the digestive cycle corresponding to the continuous influx of alkaline fluid from the pancreas. These quantitative changes in the outflow of the juice were observed in certain of the bile experiments, referred to above.

In the work hitherto reported the activity of the stomach, as far as it concerns this phase of the self-regulating mechanism, has only been followed by the introduction into the stomach of a given quantity of some alkaline solution. These experiments, however, afford us an opportunity to watch the reactions of the stomach to alkaline material repeatedly administered throughout the digestive cycle.

The results obtained serve to draw attention to the remarkable capacity of the stomach to maintain a relatively high acidity of its contents. In the past perhaps undue emphasis has been laid on the occurrence of duodenal regurgitation. We shall probably be more exact in our conception of the mechanism which controls the level of gastric acidity when we constantly bear in mind that it, in turn, depends upon two more or less distinct, subsidiary mechanisms. One of these concerns the neutralization of hyperacid chyme through duodenal regurgitation. The other, a gastric process, provides for an outpouring of gastric juice when the stomach contents are below the normal acidity.

These findings have more or less clinical significance since it is not at all certain, at present, how much of the favorable results obtained from the medical treatment of gastric ulcer is due to a lowering of the acidity level. Carlson (1917) suggests that the relief from pain following the administration of alkalis or following gastroenterostomy may be due to other factors than the effect on the gastric juice. These factors may also explain, in part, the excellent results observed after the Finney pyloroplasty since this operation affords ideal conditions for the entrance of duodenal juices into the stomach.

The possibility, of course, must be borne in mind that subsequent to the transplantation of the duct into the stomach wall, the pancreas may have ceased discharging juice. Against this assumption, however, there is more or less conclusive evidence. In the first place, the results from the analyses indicate that there was a slight depression of the acidity level at the 3 hour period of digestion after transplantation of the duct, a finding to which there was no exception among the seven animals used. This pointed to an alteration in the chemistry of the stomach. In the second place, histological study of the parts of the pancreas adjacent to the large duct disclosed no discernible pathological changes such as would be expected were the duct occluded. In Hess' experience ligation of the ducts of the pancreas leads to a sclerosis of the entire gland. The amount of sclerosis is proportional to the size of the duct which has been ligated, together with the number of its communications. What is more, special stains demonstrated that the cells in this neighborhood were charged with secretion granules and, accordingly, that they were in an active



state. In the third place, the results obtained from the trypsin determinations pointed toward the presence of an unusual quantity of pancreatic juice in the stomach subsequent to the transference of the duct from the duodenum to the stomach. And, finally, dissection of the specimens removed at necropsy showed in each instance a patent duct opening into the small duodenal transplant, with a spacious communication between the latter and the stomach. In one animal the postmortem examination was made about 120 days subsequent to the operation on the pancreas.

The most convincing evidence that pancreatic juice is regularly discharged into the stomach after this plastic operation is afforded by the results which have been obtained in some more recent experiments concerning duodenal extirpation. While this work is still incomplete it has nevertheless shown that in duodenectomized animals where the major pancreatic duct has been transplanted into the gastric wall the pancreas remains histologically normal at the end of the 5th week. For reasons which will be discussed in a subsequent communication these animals have succumbed at about the end of that period. The fact, however, that no changes of an atrophic nature were ever discovered in the pancreas at postmortem examination, together with the invariable finding of a definite gastritis, furnish reliable evidence that the pancreas continued to discharge its external secretion into the digestive tract through its new communication with the stomach.

Making use of a modification of Ehrenreich's test, Spencer, Meyer, Rehfuss, and Hawk studied the trypsin values of the gastric contents in man under varying conditions. The results of their experiments serve to substantiate the regurgitation hypothesis of Boldyreff, and indicate that tryptic digestion frequently proceeds in the normal stomach. In view of this work an attempt was made to follow the trypsin values of the stomach contents from two dogs (Nos. 3 and 7) both previous and subsequent to the operation on the pancreas. The test was repeated a considerable number of times with each animal.

Practically no digestion was ever noted in the tests carried out on normal animals. Following the transplantation of the major pancreatic duct into the stomach, however, there was a definite digestion of casein. A certain variation in the tryptic activity of the gastric

contents was appreciable from day to day, but this did not serve to obscure the difference referred to.

The new conditions established by the transplantation of the duct probably led to definite changes in the rate and character of digestion, both in the stomach and in the duodenum. Many believe that gastric juice is peculiarly destructive to the pancreatic ferment by virtue of the action of hydrochloric acid on the enzyme. As Long and Hull have shown, however, this may not actually be the case. Some tryptic digestion may occur within the stomach provided the free acid remains sufficiently low through protein combinations. Interesting as this aspect of the problem is, no further observations concerning digestion were carried out.

The animals were kept under observation for varying periods. Dog 7 was killed 121 days subsequent to the transplantation of the pancreatic duct. At that time it was in perfect health, having weaned a litter of well nourished pups a few days previously. In the series of seven dogs there was one death (Dog 1). This animal was active and appeared to be in good condition up to a few days before it died. At autopsy no satisfactory cause of death was discovered. There were many intestinal worms, but, as most of the laboratory animals harbor parasites of this nature, the findings may have no practical significance. Of the remaining five animals three were moderately well nourished and two rather undernourished when they were killed. All of them, however, ate heartily of their food.

Postmortem examination revealed an interesting condition at the site of the transplantation. In each animal there was a tiny pouch of duodenum attached to the stomach. The line of demarcation between the two was clearly evident, and as far as the macroscopic features were concerned, there was little to differentiate the transplant from the neighboring duodenum. It retained some, if not all, of its original blood supply.

The major pancreatic duct was readily found opening into the base of the pouch. Inspection of the wall showed it to be soft and of normal caliber, and on instrumentation the duct admitted a relatively large probe. There were no signs of atresia of the duct in any of the seven specimens examined. A search was made in each case for any possible accessory communications between the gland and the duo-

denum. We know from the studies of Hess on the canine pancreas that there may be three and occasionally four ducts opening into the duodenum. The main duct which arises from the union of two large branches drains the principal canal system of the pancreas. The accessory duct lies more oralward and opens into the intestinal lumen in association with the ductus choledochus. Between the two lies a smaller communicating channel. A fourth duct may connect the pars descendens with the duodenum.

In none of the specimens was the accessory duct found. But the search was not complete in any case since it was necessary to preserve the structures for histological study. In two of the seven animals a very small communication was found opening into the duodenum just proximal to the site of the end to end anastomosis. If a third duct of this type had been present in the other animals it seemed probable at the time of the necropsies that it had been destroyed in the course of the transplantations and the anastomoses.

The observations which have since been made on the duodenectomized animals, however, indicate clearly that some accessory communication between the pancreas and the intestine must have existed in each dog of the series, since the discharge into the stomach of the total output of pancreatic juice leads to profound changes in nutrition and to the development of a definite gastritis, and evidences of these processes were never discovered in any of the animals under discussion.

In gross the pancreas seemed normal in every way except that it gave the impression of being somewhat smaller than usual. The mucosa of the duodenal transplant, of the stomach, and of the intestine throughout its length showed no signs of ulceration or sclerotic changes. There was no injection even in the vicinity of the pancreatic duct. Lönnqvist has reported a temporary catarrhal condition of the gastric mucous membrane following a profuse reflux of bile and pancreatic juice into the stomach. There was nothing, however, either in the anatomical findings or in the characteristics of the samples of test meal removed to suggest this condition in any of these animals. But the experiences with duodenectomized animals have since demonstrated that pathological changes make their

appearance in the stomach as soon as the total quantity of the pancreatic secretion is diverted into the viscus.

Histological study of the pancreas disclosed no inflammatory or degenerative changes. It should be emphasized, nevertheless, that sections were prepared only from the central portions of the gland. Had the tissue adjoining the lesser duct been studied it is possible that the examination might have revealed some atrophic changes. In four of the specimens sections were prepared with the acid fuchsin-methylene green stain of Bensley. With this technique the cells were seen to be filled with secretion granules. The preparations examined were considered to represent normal tissue.<sup>1</sup>

The urine of one animal was followed for a number of weeks subsequent to the second operation (transplantation of the pancreatic duct). While some reducing substances were occasionally encountered the findings at no time suggested the presence of sugar.

#### SUMMARY AND CONCLUSIONS.

The mechanism described for maintaining the optimum level of gastric acidity is designated by Boldyreff as the "self regulation of the acidity of the contents of the stomach." In support of Boldyreff's hypothesis is the evidence obtained from many experiments carried out both on man and on animals, in which solutions of alkali and acid have been placed in the stomach. The introduction of acid fluid has led to a regurgitation of alkaline duodenal contents, whereas the introduction of alkaline solutions has called forth a secretion of acid gastric juice.

The experiments reported in this paper were carried out for the purpose of ascertaining how the stomach would react, in as far as the secretion of hydrochloric acid is concerned, to a more or less continuous influx of relatively strong alkaline fluid, prolonged throughout the cycle of digestion. Numerous studies have shown that any serious interference with the process of regurgitation leads to a rise in the acidity level of the stomach; *i.e.*, to a state of hyperacidity. There is but little evidence, however, to indicate whether the acidity level will be depressed temporarily or permanently (hypoacidity)

<sup>1</sup> Dr. E. Goetsch examined these preparations.

when alkaline material, in considerable amounts, continues to enter the stomach.

The influx of alkaline fluid was provided for by transplanting the larger pancreatic duct into the wall of the stomach after ligating and dividing the lesser duct. Specimens of test meal for analysis were withdrawn through gastric fistulas made after the method of Janeway.

Animals prepared in this manner served also to furnish additional information regarding the possible relation of the hydrochloric acid of the gastric juice to certain acute inflammatory and chronic sclerotic changes in the pancreas.

From the results of these experiments it appears that the presence of a considerable amount of pancreatic juice in the stomach throughout the period of digestion leads only to a moderate decrease in the acidity level of the ingesta in the later stages of digestion. Earlier in the process there is no constant alteration of the acidity level in either direction. The findings then serve not only to corroborate the views of Boldyreff, but also to demonstrate the remarkable compensatory activity of the gastric glands under conditions which entail an unusual quantity of alkali in the stomach.

In addition the work has shown that when the larger pancreatic duct is properly transplanted into the wall of the stomach, it may remain patent for months. In animals in which this operative procedure has been carried out, the pancreas has been found to undergo no inflammatory or other degenerative changes. This finding is regarded as evidence against the postulation of Hlava that gastric juice is probably responsible for the occurrence of certain cases of acute hemorrhagic pancreatitis.

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# THE ABIOTIC ACTION OF ULTRA-VIOLET LIGHT.

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PLATE 66.

(Received for publication, July 14, 1917.)

## INTRODUCTION.

The following data as to the bactericidal action of ultra-violet light on typhoid bacilli were obtained by a method different from that heretofore used and the results give additional evidence toward settling certain disputed phases of the subject.

In 1910<sup>1</sup> and again in 1912<sup>2</sup> Victor Henri and his associates published articles on this subject in which they give evidence to show that the bactericidal effect of ultra-violet light first becomes appreciable at 3000 Ångström units, and increases progressively from there downward toward 2150 Ångström units. The amount of this progressive increase they did not make clear. The evidence tended to show that there was no maximum in this region. The organisms were exposed directly to the ultra-violet light source, so arranged as to limit to a certain extent its spectral range. This limitation was effected by choosing three sources of ultra-violet light such that their intensities in various regions were quite different, and to emphasize further this difference the spectral distribution of their radiation was cut down by the interposition of one of six light-screens. It is difficult from the tables of data to follow them to their conclusions. The upper limit for the action of ultra-violet light they fixed by screening out the lower wave-lengths with glass and determining the amount of screening necessary to prevent the effect.

Browning and Russ<sup>3</sup> state that they prepared plates by making a thin spread of bacteria on glass-supported films of nutrient material. These were exposed in

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<sup>1</sup> Cernovodeanu, P., and Henri, V., *Compt. rend. Acad.*, 1910, cl, 52, 549.

<sup>2</sup> Henri and Henri, *Compt. rend. Acad.*, 1912, clv, 315.

<sup>3</sup> Browning and Russ read a paper on this subject recently before the Royal Society which has not yet appeared in print. Their reading is abstracted, however, in the *Brit. Med. J.*, 1917, i, 656.

As this proof goes to print the paper of Browning and Russ has appeared (Browning, C. H., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917, xc,<sup>4</sup>

the manner of a photographic plate at the back of a quartz spectrograph. The sterilizing action of the light gave a photograph of the active portion of the spectrum. They fixed 2960 Ångström units as the upper limit of effective light.

Lyman<sup>4</sup> has shown that the abiotic effect of ultra-violet light increases rapidly below 2000 Ångström units to become almost instantaneous at 1750. The lower limit of sensitivity has not been fixed. I have shown in a previous paper<sup>5</sup> that typhoid bacilli are slightly sensitive in the x-ray region (1 Ångström unit). There may, however, be one or more minima between 1 and 1750 Ångström units.

### *Method.*

My experiments differ from previous ones in that water is used as a medium for the organisms. Experiments with the x-rays and with chemicals seem to show that nutrient media often protect the organisms from the action of abiotic agents. My experiments further differ from those of Henri and his associates in that the light incident on the organisms is confined in each case by the use of a quartz spectrograph to a very narrow region of the spectrum.

In the following experiments I have used a fresh water suspension of typhoid bacilli. A uniform quantity of the suspension was taken up on a platinum loop and used to fill a quartz capillary by capillary action. The quartz capillary was then fixed in an appropriate graduated rack in the focal plane of a quartz spectrograph and so

33). They found the cessation of abiotic action to be sharply at 2960 Ångström units for *Staphylococcus aureus*, and slightly higher, at 3000 Ångström units, for *B. typhosus*. An excellent plate for *Staphylococcus aureus* accompanying the article would indicate that the fall in abiotic action becomes perceptible at 2900 Ångström units and comes to zero at 2960 Ångström units. My quantitative data, graphically illustrated in Text-fig. 1, show that this fall to 2960 Ångström units is not so sharp as their plate would lead one to suppose. The probability that long exposures to sunlight have a killing effect would indicate that the curve of the figure approaches the horizontal axis asymptotically, that the curve is concave to the right as shown, rather than convex to the right, as an abrupt fall to zero would imply. My actual numerical data further imply this concavity. Browning and Russ required much longer exposures to kill the organisms than I did. My spectrograph is the same as theirs and my iron spark is probably not brighter than the tungsten arc which they used. The differences in exposure required by us would therefore seem to offer interesting corroborative evidence as to the protective effect of a nutrient medium for bacteria.

<sup>4</sup> Lyman, T., *Spectroscopy of the extreme ultra-violet*, London, 1914, 103.

<sup>5</sup> Newcomer, H. S., *J. Exp. Med.*, 1917, xxvi, 657.



exposed to a narrow region of the spectrum. After exposure the contents of the capillary were washed out and rinsed by suction in liquid agar and plated, the plates incubated, and counts made. The quartz spectrograph was a Hilger size C, giving a spectrum from 2100 to 8000 Ångström units 19 cm. long. The focal plane of the spectrograph formed an acute angle of about  $30^\circ$  with the light path. For most of the work the condensed iron spark was used as an ultra-violet source. It was placed 10 cm. from the entrance slit of the spectrograph. It was operated by a 10 kilowatt closed circuit transformer using 110 volts alternating current on the primary. The secondary voltage was about 10,000. The condensers had a capacity of 0.05 microfarad. The entrance slit of the spectrograph was opened to 1.5 mm., a width such that the individual lines of the iron spark were spread out into bands of the same width in projection as the quartz capillary. This produced an overlapping of the lines, giving the effect of a continuous spectrum. As a result any setting involved an exposure to a wave-length interval 15 to 40 Ångström units long. Fig. 1 reproduces a photograph of the iron spark spectrum, the upper band being the spectrum with a narrow slit, and the lower, the spectrum as used with a wide slit. The figure gives some information as to the relative intensity of light at different wave-lengths.

My data give the approximate wave-length range of the light falling on the quartz capillary for each setting. These wave-lengths were determined by measurements with a wave-length scale on photographs of the spark spectrum, the rack holding the capillaries being graduated to correspond with the wave-length scale. No attempt is made to give wave-lengths closer than to within 5 Ångström units. The data are computed on a basis of controls having counts of 100 colonies per sq. cm. The actual controls varied from 10 to 800 per sq. cm., usually about 150. When the controls gave more than 1,000 per sq. cm. the suspension contained so many organisms that some of them were protected from the light by being in the shadow of others.

## OBSERVATIONS.

Table I gives the data for 10 and 5 minute exposures to the iron spark.

TABLE I.

*Exposures to the Iron Spark of 10 and 5 Minutes' Duration.*

Wave-length.	10 min. exposures.	5 min. exposures.	Wave-length.	10 min. exposures.	5 min. exposures.
<i>A. u.</i>			<i>A. u.</i>		
1090-2005	41*	73	2530-2560		0
1095-2110	50	62	2540-2570	0	3
2195-2215	8		2550-2580	0	
2205-2225	11		2560-2590	0, 0	$\frac{1}{2}$
2235-2255	25		2580-2610	0	0
2240-2260	28	50	2600-2630	0	$\frac{1}{4}$
2250-2270	65		2610-2640	0	0
2270-2290	30		2645-2675	1	9
2280-2300	26		2650-2680	$\frac{1}{2}$	1
2290-2310	2, 22	51	2655-2685	$1\frac{1}{2}, 1\frac{1}{2}$	$3\frac{1}{2}$
2325-2350	$2\frac{1}{2}$	4	2680-2715	$0, \frac{1}{2}$	3, 3
2330-2355	1	14	2700-2735	0	1
2335-2360	$2\frac{1}{2}$	3	2760-2795	0	
2350-2375	$3\frac{1}{2}$	11	2780-2815	0	9, 10
2365-2390	$\frac{1}{2}$	$1\frac{1}{2}$	2785-2825	1	
2385-2410	$0, \frac{1}{2}$	$0, 1\frac{1}{2}, 3$	2790-2830	2	5
2405-2430	$\frac{1}{10}$	$1\frac{1}{4}$	2795-2835	$1\frac{1}{2}$	$4\frac{1}{2}$
2435-2460	0	2	2845-2885	7	
2455-2480	1	$2\frac{1}{2}$	2850-2890		16, 17, 20
2485-2510	0, 0	0, 1, 1	2855-2895	$3, 2\frac{1}{2}$	$5, 7\frac{1}{2}, 7\frac{1}{2}$
2495-2525	0	1, 1, 2	2875-2915	7	
2500-2530	0	0	2945-2985	40, 45, 50	
2525-2555	0	$\frac{1}{4}$			

\* In the tables the data represent typhoid organisms per hundred remaining alive after the given exposure.

It is to be noted that the abiotic effect is low from 2100 to 2300. This corresponds to a portion of the iron spark spectrum of relatively weak intensity. In order to determine the nature of the effect in this region exposures were made to the copper and zinc spark, sources with lines of about equal intensity in this and higher regions of the spectrum. Table II gives the results of these exposures and shows that the effect is about the same in this region as elsewhere.

TABLE II.  
*Exposures to the Copper and Zinc Sparks.*

Copper spark.		Zinc spark.		
Wave-length.	10 min. exposures.	Wave-length.	10 min. exposures.	5 min. exposures.
$\text{\AA. u.}$		$\text{\AA. u.}$		
2130-2140	57, 22	1990-2105	1	14
2180-2195	30, 23	2130-2145	5	21
2205-2225	30	2190-2210	37	
2235-2250	32	2250-2270	37	61
2265-2290	47	2490-2530*		1
2295-2320	79, 81	2545-2575*		0
2360-2385	24	2800-2840*	2	1½
2590-2620	47, 63			
2750-2785	49			

\* Very strong lines.

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#### ERRATUM.

On page 844, Vol. XXVI, No. 6, December 1, 1917, Table I, Column 1, for *Wave-lengths,  $\text{\AA. u.}$ , 1990-2005 and 1995-2010*, read *2090-2105 and 2095-2110*.

On page 845, Table II, Column 3, for *Wave-lengths,  $\text{\AA. u.}$ , 1990-2105* read *2090-2105*.

2365-2390	13, 12, 13	2580-2610	1, 0, 1, 0
2385-2410	16, 20	2680-2715	22, 20, 21
2435-2460	13, 12½, 12	2850-2890	45, 52, 45
2530-2560	4, 2, 2, 1½, 1		

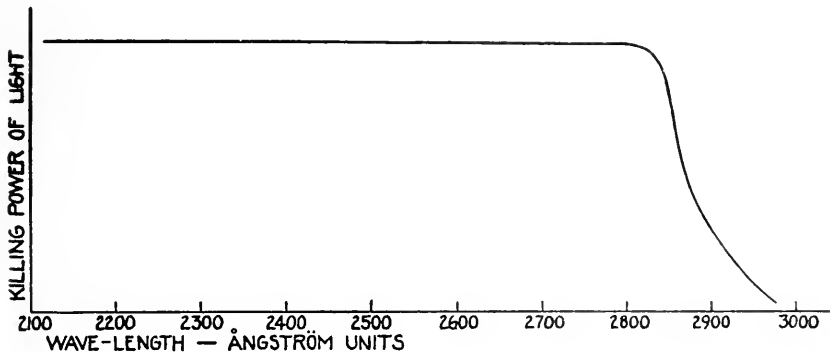
From Table III the decrease would appear to begin at 2700, but at this point the iron spark spectrum is rather weak. At 2750 the spectrum is very strong and here exposures corresponded with those made at 2600, also a relatively strong place in the spectrum. The

iron spark spectrum is slightly weaker below 2500 than above that point. Below 2320 it is very weak. Above 2780 it weakens perceptibly. About 2870 corresponds to a region of the spectrum equal in intensity to the 2700 region. At 2870 the tables show a decrease in abiotic activity. This decrease when corrected for the weakness of the spectrum amounts to a fall of one-half. The last appreciable abiotic effect occurs somewhere between the moderately strong 2950 and 2990 lines. In this region the intensity is about the same as at 2700 and 2870. At 2945 to 2985 exposures for 10 minutes averaged 55 per cent killed, for 20 minutes 81 per cent killed. An average of eight exposures at 2990 to 3030 for 20 minutes gave no mortality. These exposures at 2945 to 2985 and 2990 to 3030 were controlled for evaporation and for scattered light. Longer exposures were impracticable because of the increasing influence of both these factors, particularly the former. Complete evaporation of the contents of the capillary occurring in about 40 minutes will completely or almost completely kill the typhoid organisms.

In order to limit further the upper end of the abiotic spectrum exposures were made directly before the iron spark but behind a piece of optical glass 3 mm. thick. The distance from the spark was 4 cm., the increase in energy due to lessened distances alone amounting to 256 times. Besides this, the whole area of the spark was effective rather than a portion corresponding to the slit area. This introduced another factor so that there was an increase of about one thousandfold. The glass transmitted no light below 3100; above 3300 it was almost transparent. These exposures of 5 and 10 minutes' length had no effect on the typhoid organism. The terrestrial sun's spectrum ends somewhere near 2970. Exposures to sunlight of 20 minutes' duration produced no effect.

I, therefore, conclude that the killing power of ultra-violet light begins to decrease at about 2800, at first very gradually, reaches one-half of its value at about 2870, one-tenth of its value at 2950, and less than one one-hundredth at 2990. Text-fig. 1 gives a sketch of the probable form of the abiotic curve. As far as I can judge equal intensities produced equal effects in the region 2100 to 2800. If there is a maximum in this region it is at the most only slight and would be in the neighborhood of 2600.

The relative effect of much shorter exposures is shown in Table IV. These exposures give at the same time further data for determining the relation between quantity of energy and per cent of organisms killed. This relation, most likely an integral of the probability law, must be known in order to derive from the tables the form of the abiotic energy curve of Text-fig. 1.



TEXT-FIG. 1. Graph representing the approximate amount of abiotic energy in ultra-violet light as referred to the typhoid bacillus.

TABLE IV.  
*Short Exposures to the Iron Spark.*

Time.	Wave-length.	
	2385-2410 Å. u.	2580-2610 Å. u.
<i>sec.</i>		
60	14, 16, 14, 12	1, 5, 6
50		2
40		3, 3, 2
30	36, 24, 12	5, 4, 5
20		11, 7, 8, 4, 10
15	72, 50, 42	
10		12, 25, 11, 12, 17, 40

The Wratten and Wainwright panchromatic plate requires an exposure of about  $\frac{1}{4}$  second under these conditions. At 2600 a 95 per cent sterilization of a *Bacillus typhosus* suspension is secured in about 200 times this length of time. The typhoid organism there-

fore compares very well with the slow silver papers in degree but not in range of sensitivity.

In similar but less extensive experiments I have found *Staphylococcus aureus* to behave exactly like *Bacillus typhosus*.

#### CONCLUSIONS.

Typhoid bacilli are about one two-hundredth as sensitive to ultra-violet light of wave-lengths 2100 to 2800 Ångström units as is the photographic plate. This sensitivity then falls off, decreasing rapidly to almost zero sensitivity at about 2970, the beginning of the sun's spectrum.

I wish to thank Dr. Paul Lewis for suggestions and advice in the arrangement of the experiments.

#### EXPLANATION OF PLATE 66.

FIG. 1. The iron spark spectrum between 2100 and 3200 Ångström units. The upper band is the spectrum with a narrow slit, the lower band, the spectrum with a wide slit as for bacterial exposures. Below 2320 Ångström units the faintness of the spectrum is real. Below 2200 the increasing faintness is due to the plate.



FIG. 1.

(Newcomer: Abiotic action of ultra-violet light.)





# CHANGES IN THE PERIPHERAL BLOOD CONSEQUENT UPON THE DIVERSION OF THE SPLENIC BLOOD INTO THE GENERAL CIRCULATION.

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PLATE 67.

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Studies of the peripheral circulation after Eck fistula in dogs have been reported, but on account of the fact that by diverting the portal blood into the inferior vena cava one has included blood from all the tributaries of the portal vein, it seemed advisable to make a thorough and careful study after the splenic blood alone had been thrown into the general circulation.

## HISTORICAL.

A review of the literature revealed the fact that numerous investigators had attempted to discover the function of the spleen by means of a study of the splenic circulation itself. Before Eck, in 1877, devised his method of diverting the portal and splenic blood into the general circulation, Dobson, Warner, Eagle, Hargrave, and others had advanced various theories in regard to the function of the spleen that were based upon the distribution of the splenic circulation as determined by anatomical dissection in animals and in human cases. Ecker and Gerlach studied the blood corpuscles in relation to the spleen. And in 1851 Funke made physiological and chemical experiments upon the blood of the splenic artery and vein. Following the publication of the experimental method and the results of diverting the portal circulation into the inferior vena cava by Eck in 1877, extensive studies have been made on animals with an Eck fistula. That most of these studies were physiological and chemical is evident from reading the reports of Hahn, Massen, Nencki, and Pawlow, Kotliar, Hedon and Delezenne, de Filippi, Schupfer, Magnanini, Salaskin and Zaleski, Popelski, Bielka von Karltru, Tansini, Rothberger and Winterberg, Hawk, Macleod, Sweet and Levene, Voegtlin and Bernheim, Towles and Voegtlin, Matthews, Fischler and Kossow, and Whipple and Hooper. A number of different methods of per-

forming the anastomosis between the portal vein and the inferior vena cava, with the purpose in view of improving the operative technique, have been reported by Pawlow and Massen in 1893, by Queirolo in 1895, by Sweet and Herrick in 1905, by Guleke in 1906, by Fischler and Schröder in 1909, by Bernheim, Homans, and Voegtlin in 1910, by Frouin and Jéanne in 1911, by Franke in 1912, by Jeger and Reschad and du Bois-Reymond in 1913, and by Peet in 1914.

A study of the peripheral blood after Eck fistula was made by Nassau in 1914, who concluded from his investigation that the blood picture of dogs with Eck fistula did not deviate from the normal, neither did the tissue intoxication or the

TABLE I.

Author.	Red blood count.	White blood count.	Percentage of white blood count.		
			Polymorpho-nuclear neutrophils.	Polymorpho-nuclear eosinophils.	Mononuclears.
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Schittenhelm, Weichardt, and Grisshammer.	5,500,000–6,000,000	9,000–12,000	63–77	3–5	17–25
Kleinberger.	7,225,000	10,000	77.3	4.2	8.6* 7.0†
Nassau.	5,400,000–7,300,000	5,000–13,400	63–79 (72)	3.5–10	13–25.5 (18)* 2.67–6.6 (5.2)‡
Musser and Krumbhaar.	4,630,000–7,760,000	15,923	66	5	22* 6.8†

\* Small mononuclears.

† Large mononuclears.

‡ Large mononuclears and transitionals.

anaphylactic reaction. In dogs with Eck fistula he was unable after repeated injections of albumin to demonstrate the classical blood picture of anaphylactic shock. Also dogs with Eck fistula and ligation of the common bile duct became jaundiced and died after a short time in the course of the jaundice, and the red blood cell count dropped within 2 to 6 days approximately to two million cells. And finally, the resistance of the red blood cells of Eck fistula dogs against hypotonic common salt solution was unchanged. Table I shows the normal blood count of dogs as determined by Nassau and others.

There was also quite a variation in the blood counts of normal dogs, as was given in the reports of observations by Busch and Van Bergen, Dawson, Goodall,

Biedel and Decastella, Tallquist and Reckzeh.<sup>1</sup> It was therefore evident that the most accurate method to employ in order to avoid this great variation in animals would be to determine the control blood counts of each dog that was studied.

A search through the literature revealed only one instance in which a study of the peripheral circulation after the splenic blood alone had been diverted into the general circulation is given; and the authors, Krumbhaar, Musser, and Peet, reported difficulty in doing the anastomosis between the splenic vein and the inferior vena cava. Following the operation they noted an immediate polymorphonuclear leukocytosis and transitional rise, which was prolonged by a more persistent lymphocytosis and eosinophilia, as in splenectomized animals; also a moderate anemia and slight increased resistance of the red blood cells to hypotonic salt solution; a postoperative loss of weight followed by an increased weight, as in splenectomized animals; abdominal adhesions and perisplenitis; and the urine free from bile.

#### EXPERIMENTAL.

Because of the rather uniform results obtained in the five large dogs that were studied and the simple method that was used to divert the splenic blood into the general circulation, it seemed advisable to report the following experiments. The method employed in the present problem consisted in the study of five dogs over a sufficiently long period before operation in order to establish accurate controls for each dog; and after the splenic blood had been diverted into the general circulation this study was repeated over a period varying from 1 to 4 months' duration in the different animals. In addition, 2 weeks before the end of the experiment the dogs were vitally stained to a maximum with trypan blue, and finally they were killed in order to determine the gross and histological findings.

At the time of operation, with the animals under ether anesthesia, the spleen was first measured through a left rectus incision and a small histological control section removed. After the splenic and the left renal vein had been dissected out, and the common trunk of the splenic vein had been divided between double ligatures, a lateral anastomosis was made between the renal and splenic veins, using fine beaded black silk, oil technique, and a Peet three-bladed, spring-jawed blood vessel clamp; and lastly, all possible collateral circulation

<sup>1</sup> These authors are quoted by Musser and Krumbhaar in their article on studies of the blood of normal dogs (1914-16).

with the spleen was divided, especially the communicating branches between the spleen and the stomach (Fig. 1).

The animals recovered rapidly from operation and the wounds healed by first intention in every case. The dogs kept in splendid general condition throughout the experiment and several of them were operated upon a second and a third time in order to verify intra-

TABLE II.  
*General Blood and Weight Table of Dog I.\**

Date.	Average of two white blood counts.	Average of two red blood counts.	Hemoglobin (Sahli).	Weight.
1917			<i>per cent</i>	<i>lbs.</i>
Apr. 11	17,180	8,424,000	83	31 $\frac{3}{8}$
" 12	14,700	8,228,000	86	30 $\frac{3}{8}$
" 13	16,100	7,856,000	87	31 $\frac{1}{2}$
" 21	Date of operation.			.
" 22	45,200	8,644,000	93	28 $\frac{5}{8}$
" 23	36,900	7,812,000	83	28 $\frac{7}{8}$
" 24	30,540	7,952,000	83	29
" 25	31,460	7,908,000	86	29 $\frac{1}{4}$
" 26	21,720	8,164,000	81	29
" 27	19,220	8,084,000	89	29 $\frac{3}{8}$
" 28	33,480	8,000,000	90	28
" 30	27,070	8,384,000	87	28 $\frac{1}{8}$
May 2	24,560	7,748,000	84	28
" 4	25,980	7,864,000	78	27 $\frac{3}{4}$
" 7	22,580	7,890,000	79	28
" 9	15,120	7,644,000	91	27 $\frac{3}{4}$
" 11	19,020	7,921,000	83	27 $\frac{7}{8}$
" 14	20,340	7,196,000	85	28
" 19	16,040	7,616,000	86	30 $\frac{1}{8}$
" 21	16,960	7,732,000	83	30

\* See Text-fig. 1 for graphic chart.

abdominal conditions, with the result that no collateral circulation and no intraabdominal adhesions were found, and the anastomosis was working. Fig. 1 clearly shows the anatomical arrangement and the final operative result which occurred in every instance. Aside from a moderate temporary loss of weight after operation, nothing unusual was noted in the condition or actions of the animals at any

time. The animals were never jaundiced and the urine and stools were negative.

Tables II to V and Text-figs. 1 to 4 show most clearly and briefly the general condition of the animals and the changes in the peripheral blood. Because of lack of space and also since all the animals showed essentially the same changes, only a few tables and charts from two

TABLE III.  
*General Blood and Weight Table of Dog 2.\**

Date.	Average of two white blood counts.	Average of two red blood counts.	Hemoglobin (Sahli).	Weight.
<i>1917</i>			<i>per cent</i>	<i>lbs.</i>
Mar. 2	16,580	7,620,000	88	28 $\frac{3}{8}$
" 3	18,790	7,300,000	80	28 $\frac{1}{2}$
" 4	19,800	7,204,000	80	27 $\frac{1}{2}$
" 16	Date of operation.			
" 17	28,580	7,072,000	75	27 $\frac{3}{8}$
" 18	27,420	6,768,000	70	26 $\frac{1}{16}$
" 19	22,240	7,252,000	73	25 $\frac{3}{4}$
" 20	18,060	6,732,000	78	25 $\frac{3}{8}$
" 21	14,080	6,784,000	83	27 $\frac{1}{4}$
" 23	15,760	6,280,000	72	26 $\frac{5}{8}$
" 26	10,100	6,632,000	76	25 $\frac{3}{4}$
" 30	10,760	7,533,000	82	27 $\frac{1}{8}$
Apr. 2	12,820	7,320,000	82	26 $\frac{3}{4}$
" 9	12,060	8,115,000	84	26 $\frac{5}{8}$
" 16	9,000	7,956,000	80	25 $\frac{7}{8}$
" 23	11,850	7,556,000	90	26 $\frac{1}{4}$
" 30	11,700	7,480,000	88	26 $\frac{3}{8}$
May 7	12,000	7,636,000	90	26 $\frac{3}{8}$
" 14	12,080	7,144,000	89	26 $\frac{3}{8}$
" 21	11,460	7,440,000	86	26 $\frac{3}{8}$

\* See Text-fig. 3 for graphic chart.

dogs are included in the present article. There was an immediate high postoperative rise of leukocytes without any especial change in the hemoglobin or the red blood cells, which had a normal appearance in fresh blood films. The white blood cells seemed to react more acutely and sensitively than one would have expected, in an uninfected wound, and out of all proportion to the splendid post-

TABLE IV.

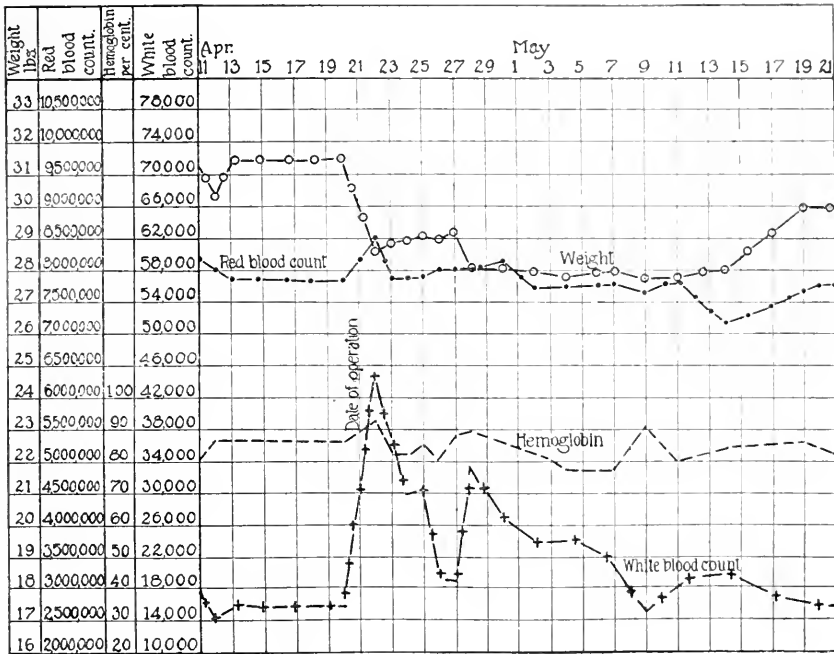
*Average Daily Differential Percentage White Blood Count Based upon Three Differential Counts of 250 Cells Each.*

Date.	Polymorphonuclear neutrophils.	Polymorphonuclear basophils.	Polymorphonuclear eosinophils.	Small mononuclears.	Large mononuclears.	Transitional cells.	Unidentified.	Smudges.
Dog 1.*								
1917	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Apr. 11	51.33	0.53	21.6	18.13	1.73	3.46	0.26	2.93
" 12	52.00	0.4	20.53	20.4	1.46	4.13	0.26	0.26
" 13	43.06	0.53	24.4	19.73	1.73	3.73	0.4	6.4
" 21	Date of operation.							
" 22	90.33	0.0	0.53	4.53	0.4	3.6	0.0	0.53
" 23	61.13	0.07	21.33	10.27	0.4	5.73	0.07	2.27
" 24	61.25	0.0	17.5	8.8	1.4	9.2	0.1	1.1
" 25	54.0	0.0	18.3	15.1	1.9	9.7	0.1	0.9
" 26	47.2	0.1	20.2	18.9	2.3	9.8	0.1	1.4
" 27	47.10	0.0	13.7	14.9	2.5	10.0	0.3	5.3
" 28	52.90	0.0	17.0	17.6	1.5	9.9	0.1	2.1
" 30	56.2	0.2	11.8	16.1	1.8	8.5	0.1	1.9
May 2	61.90	0.1	8.6	13.7	2.8	8.2	0.1	4.6
" 4	57.3	0.0	9.8	21.0	2.7	8.4	0.2	0.5
" 7	64.53	0.0	7.3	19.07	0.8	6.53	0.0	1.73
" 9	44.0	0.0	19.73	29.2	1.83	7.6	0.0	0.93
" 11	49.2	0.0	20.27	23.47	1.2	5.3	0.13	0.4
" 14	52.53	0.0	20.43	18.47	0.93	6.27	0.0	1.3
" 19	50.53	0.0	21.3	20.53	1.2	5.47	0.0	0.93
" 21	53.53	0.0	17.2	21.07	1.2	6.13	0.0	0.53
Dog 2.†								
Mar. 2	57.1	0.1	17.5	13.9	2.3	5.9	0.3	2.9
" 3	63.73	0.13	9.33	17.86	2.4	5.2	0.26	1.2
" 4	64.0	0.0	18.4	11.46	0.93	3.86	0.13	1.2
" 16	Date of operation.							
" 17	88.93	0.0	1.6	2.8	1.06	3.6	0.0	2.0
" 18	78.4	0.13	7.73	6.93	0.93	6.93	0.26	2.0
" 19	71.2	0.13	8.93	8.4	0.53	9.6	0.13	1.06
" 20	64.4	0.0	16.6	3.8	0.9	12.4	0.0	1.73
" 21	56.6	0.0	13.6	9.86	1.33	14.53	0.13	3.86
" 23	64.26	0.0	10.26	7.2	0.93	14.00	0.0	3.33
" 26	52.4	0.0	7.83	17.46	2.8	16.8	0.13	2.53
" 30	56.8	0.0	9.6	15.33	2.66	12.93	0.26	2.93
Apr. 2	48.26	0.0	16.4	18.00	2.4	11.83	0.53	2.53
" 9	58.4	0.26	20.53	28.53	2.66	11.6	0.0	1.33
" 16	42.73	0.13	21.06	19.73	2.0	12.4	0.13	2.13
" 23	40.8	0.4	28.3	16.8	2.26	9.86	0.26	0.93
" 30	41.46	0.4	28.4	14.26	2.8	10.66	0.1	1.86
May 7	41.06	0.0	23.6	19.6	1.6	9.33	0.0	1.46
" 14	44.4	0.0	24.4	18.8	2.53	8.93	0.13	0.8
" 21	49.6	0.0	26.93	13.73	1.6	7.3	0.0	0.8

\* See Text-fig. 2 for graphic chart.

† See Text-fig. 4 for graphic chart.

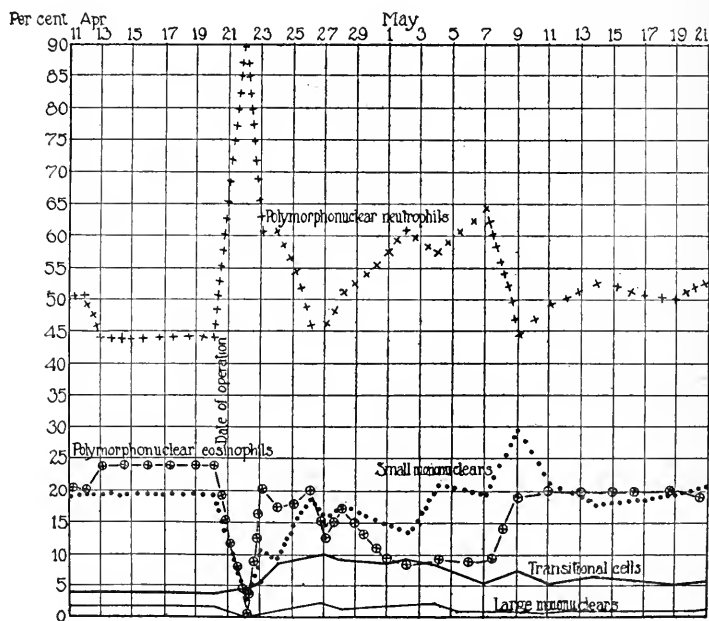
operative condition of the animals. The percentage differential white blood counts demonstrated that the great increase in leukocytes was due to the polymorphonuclear neutrophils, which composed 88 to 95 per cent of the total number of cells, and that came down to their preoperative percentage within from 2 to 7 days. But the most notable change in the peripheral blood was in the transitional white blood cells, which were immediately increased after operation,



TEXT-FIG. 1. General blood and weight chart of Dog 1 (Table II).

although they did not reach their maximum number until after 3 to 5 days. These cells, which were characterized by a large horseshoe-shaped nucleus, remained increased in number over months, and only very slowly approached their preoperative number, a fact which was probably accounted for by a gradual readjustment and decreased production to meet a lessened need in the peripheral circulation, although these transitional cells never did return quite to the preopera-

tive percentage during the course of the experiment. In several of the animals there was an immediate increase in the large mononuclear cells and so called smudges, which were probably either large mononuclear or transitional cells. The small mononuclear cells and the eosinophils were relatively decreased immediately after operation, but subsequently gradually increased to their preoperative percentage. In three animals there was a late rise of small mononuclear and eosinophilic cells with a relative decrease in the polymorphonuclear neutro-

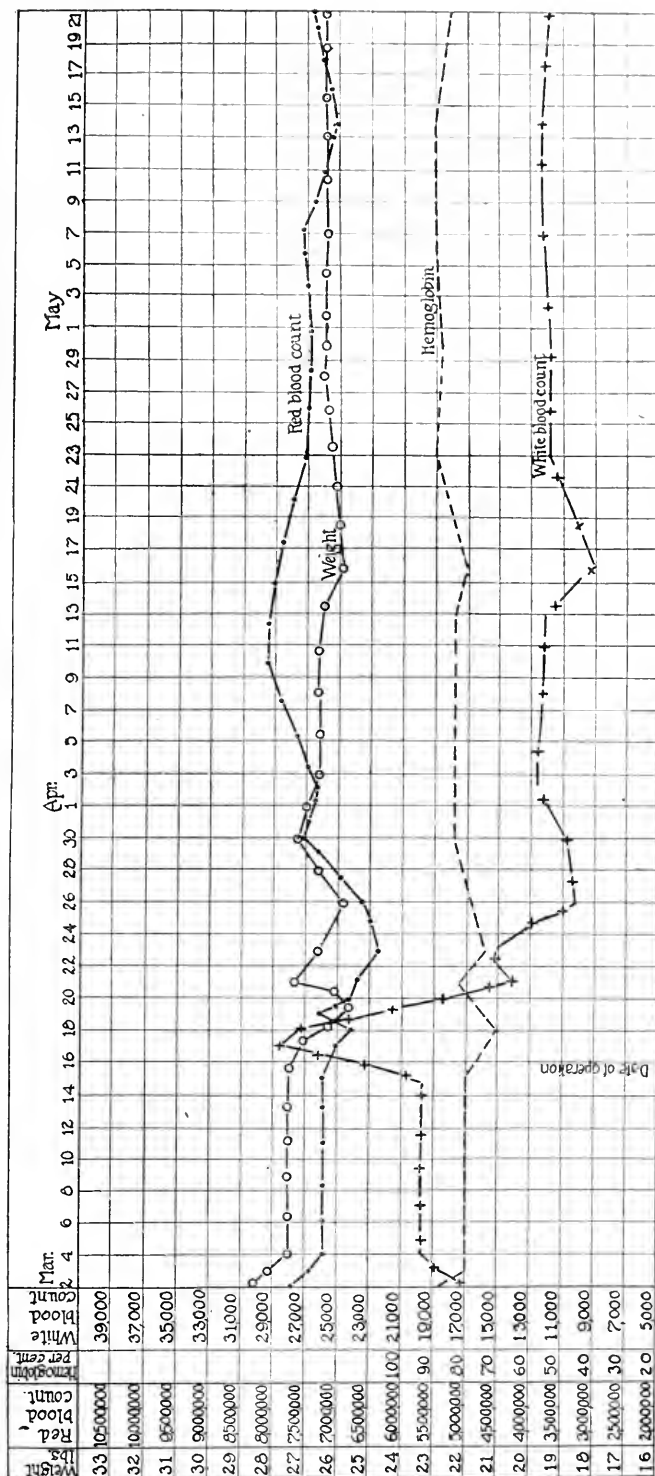


TEXT-FIG. 2. Percentage differential white blood count of Dog 1 (Table IV).

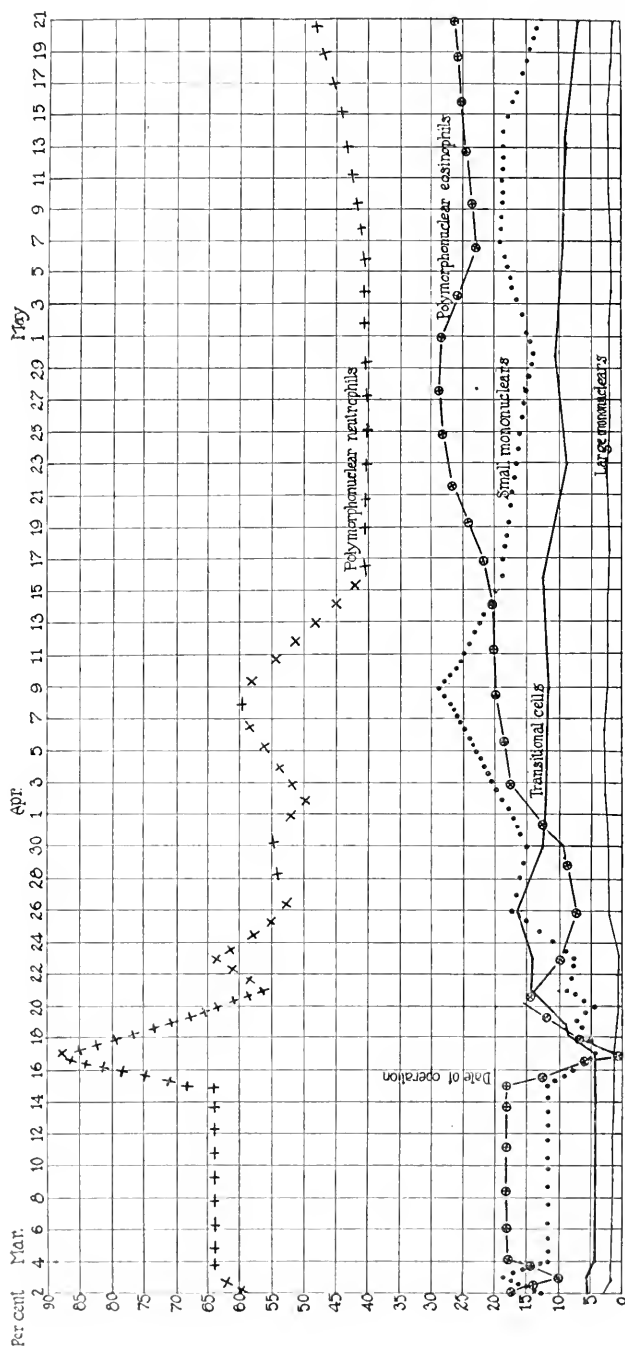
phils, and in one dog there was a late increase of small mononuclear cells alone. Naegeli gives drawings of the various types of blood cells.

After periods varying in duration from 1 to 4 months, subsequent to the first operation, the animals were killed in order to verify the splenorenal anastomosis and to note any pathological changes. The dogs were in splendid general condition, well nourished, active and healthy looking, and the tissues were well stained with trypan blue. The following procedures were carried out at the time that the ani-





TEXT-FIG. 3. General blood and weight chart of Dog 2 (Table III).



TEXT-FIG. 4. Percentage differential white blood count of Dog 2 (Table IV).

TABLE V.  
*Actual Number of Various White Blood Cells Per Cc.*

Date.	Polymorpho- clear neutro- phils.	Polymorpho- clear baso- phils.	Polymorpho- clear cosino- phils.	Small mononu- clears.	Large mononu- clears.	Transitional cells.	Unidentified.	Smudges.
Dog 1.								
<i>1917</i>								
Apr. 11	8,813.0	86.0	3,711.0	3,109.0	292.0	584.0	52.0	498.0
" 12	7,644.0	59.9	3,014.0	2,999.0	215.0	603.0	29.0	29.0
" 13	6,923.0	80.5	3,928.4	3,171.7	273.7	595.7	64.4	1,030.4
" 21	Date of opera- tion.							
" 22	40,815.6	0.0	226.0	2,034.0	180.8	1,627.2	0.0	226.0
" 23	22,545.90	36.9	7,859.7	3,800.7	147.6	2,114.4	36.9	848.7
" 24	18,690.5	0.0	5,344.5	2,686.5	427.5	2,809.68	30.54	335.9
" 25	16,988.4	0.0	5,757.18	4,744.46	597.74	3,051.6	31.46	283.14
" 26	10,251.84	21.72	4,387.44	4,005.08	499.56	2,128.56	21.72	304.08
" 27	9,052.62	0.0	2,633.19	2,863.78	480.50	1,922.00	57.66	1,018.66
" 28	17,710.92	0.0	5,691.60	5,892.48	502.20	3,314.52	33.48	703.08
" 30	15,353.84	54.64	3,223.76	4,398.52	491.76	2,322.20	27.32	529.08
May 2	15,202.64	50.00	2,112.16	3,364.72	687.67	2,013.92	24.56	1,129.96
" 4	14,786.54	24.56	2,566.04	5,455.80	701.46	2,183.32	51.98	129.9
" 7	14,564.10	0.0	1,648.34	4,312.78	180.64	1,467.70	0.0	383.86
" 9	6,652.80	0.0	2,978.64	4,415.04	272.60	1,149.12	0.0	136.08
" 11	9,357.84	0.0	3,861.06	4,569.70	228.24	1,008.06	0.0	76.08
" 14	10,678.50	0.0	4,088.34	3,762.90	183.06	1,281.42	0.0	264.42
" 19	8,100.20	0.0	3,416.52	3,288.20	192.48	882.20	0.0	144.36
" 21	9,073.60	0.0	2,917.12	3,578.56	203.52	1,034.56	0.0	84.80
Dog 2.								
Mar. 2	9,467.18	16.58	2,901.5	2,314.62	381.34	978.25	49.74	480.82
" 3	11,968.93	18.79	1,747.47	3,360.41	450.96	977.00	56.37	225.48
" 4	12,672.0	0.0	3,643.20	2,277.00	178.20	772.20	19.80	237.60
" 16	Date of opera- tion.							
" 17	25,457.62	0.0	457.28	800.24	302.94	1,028.88	0.0	591.60
" 18	21,497.28	27.42	2,111.34	1,891.48	246.78	1,891.48	82.26	548.4
" 19	15,834.88	22.24	1,979.36	1,868.16	111.20	2,135.04	22.24	244.64
" 20	11,630.64	0.0	2,997.96	686.2	162.54	2,239.44	0.0	307.02
" 21	7,969.28	0.0	1,914.88	1,393.92	183.04	2,041.60	14.08	549.12
" 23	10,143.68	0.0	1,633.28	1,134.72	14.84	2,206.40	0.0	630.08
" 26	5,292.40	0.0	787.80	1,767.50	282.80	1,696.80	10.10	252.50
" 30	6,111.68	0.0	1,032.96	2,646.28	290.52	1,388.04	32.28	312.04
Apr. 2	6,192.06	0.0	2,102.48	2,307.60	307.68	1,512.76	64.10	320.50
" 9	7,043.04	36.8	2,472.30	3,437.10	325.62	1,398.96	0.0	156.78
" 16	3,845.7	9.0	1,899.00	1,773.00	180.00	1,116.00	9.0	189.00
" 23	4,834.80	47.4	3,353.55	1,990.80	272.55	1,173.15	35.55	106.65
" 30	4,855.50	46.8	3,322.8	1,673.1	327.6	1,251.90	11.7	222.3
May 7	4,932.00	0.0	2,832.00	2,352.00	192.00	1,116.00	0.0	180.00
" 14	5,363.52	0.0	2,947.52	2,271.04	302.00	1,075.12	12.0	96.64
" 21	5,684.16	0.0	3,082.74	1,570.02	183.36	836.58	0.0	96.64

imals were killed. Under ether anesthesia, the spleen was measured and found to be either the same size or slightly larger than at the first operation. After the splenic and renal vessels were dissected out, the anastomosis was proved to be working in every case by clamping the renal vein beyond the anastomosis, which caused the spleen to swell. A probe could be passed through the anastomosis into all the various contributing branches. No gross changes were evident in the kidneys or renal veins, and there were no unusual intraabdominal adhesions and no collateral circulation established with the spleen. In two dogs the surface of the spleen was smooth and healthy in appearance. In three instances it was somewhat grayish and slightly thickened. The consistency of the spleen seemed to be the same as at the time of the first operation. In one animal the thyroid was about twice the size of those of the other dogs. In two cases the thymus was large. Otherwise, there were no gross changes in any of the organs or tissues. The gall bladder was always full of bile, and the liver seemed to be of usual size and consistency. In each instance the portal and splenic veins were tied off at several places while filled with blood, and removed for histological study. This would seem to offer a possible method of studying the blood by histological sections.

The histological study of control sections of the spleen which were removed at the time of the first operation showed them to be similar to the sections that were removed at the end of the experiment, except for a slight active congestion in the later specimens. This active congestion was the result of the method of testing the function of the anastomosis at the time that the animals were killed, and hence had nothing to do with the experimental results. There was no increase in connective tissue and the Malpighian corpuscles appeared as in the control sections. In several of the animals the cells about the portal spaces of the liver stained more deeply than the paler cells in the rest of the liver lobules. Otherwise, no changes were noted in the liver. Considerable pigment was scattered throughout the lung tissue as has been frequently observed in city-bred dogs. A routine examination of histological sections of all the remaining tissues and organs demonstrated no noteworthy changes. Special histological

studies which will be reported upon in a subsequent article are now being made upon the vitally stained spleen and other tissues.

#### SUMMARY.

1. The principal change in the peripheral blood consisted in the prolonged increase in number of the transitional white blood cells, an active brief stimulation of the polymorphonuclear neutrophils, which were later relatively decreased in number in four cases, in three dogs a late rise in number of the mononuclear and eosinophilic cells, and in one dog of mononuclear cells alone.

2. The normally high differential percentage count of polymorphonuclear eosinophils in dogs would be expected on account of the numerous parasitic infections which they usually have.

3. The splenorenal venous anastomosis offered a simple and satisfactory method of diverting the splenic blood into the general circulation because it was easy and produced no gross abnormal intra-abdominal changes, and the vessels normally lay parallel to each other and were readily approximated without tension.

4. The operation was successful in every case.

5. The animals did very well after operation and were healthy and active.

6. No noteworthy histological changes were observed in any of the organs or tissues.

7. There was no essential change in bile production that could be detected by jaundice.

8. The urine and stools showed no changes.

Finally, I wish to express my appreciation to Dr. Emil Goetsch of the Johns Hopkins University for his assistance and interest.

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## EXPLANATION OF PLATE 67.

FIG. 1. The splenorenal venous anastomosis as it appeared at the end of the experiment. The common splenic vein was divided; the gastrosplenic veins were also divided.



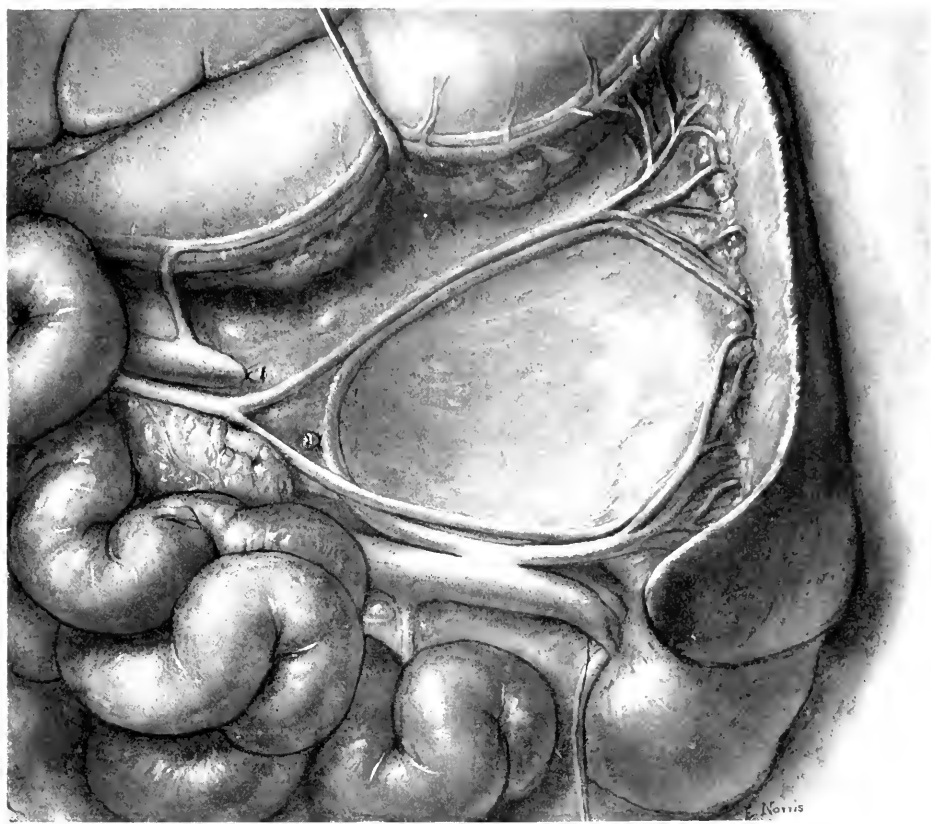


FIG. 1.

(Burket: Diversion of the splenic blood.)



# IDENTITY OF THE TOXINS OF DIFFERENT STRAINS OF BACILLUS WELCHII AND FACTORS INFLUENCING THEIR PRODUCTION IN VITRO.

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(Received for publication, September 1, 1917.)

It has been reported in an earlier publication<sup>1</sup> that under suitable cultural conditions *Bacillus welchii* produces an exotoxin which is comparable in its physical and biological properties with diphtheria and tetanus toxins. The original study was limited to five strains of the organism, all of which yielded toxins which were qualitatively identical although differing quantitatively. The identity of the various toxins was determined by the observation that an antitoxin produced with a toxin from any strain neutralized the toxins from all the other strains. Moreover, it was found that such a monovalent antitoxin would protect against and control infections with any of the strains included in the study. The small number of strains studied and the closely related sources of four of the five made it desirable to extend the experiments before concluding that all strains of *Bacillus welchii* produce the same toxin. From the standpoint of developing a specific therapy, it was essential to know whether one toxin is common to all strains, regardless of their origin. To ascertain this, twenty-two other strains have been collected from widely different sources and studied with reference to the identity of their toxins.

## *Source of the Strains.*

Two of the new strains were part of the cultures isolated during the summer of 1916 on the Western Battle Front by Dr. J. P. Simonds of Chicago. Four strains were obtained from the Pasteur Institute through the kindness of Dr. Carrel. Dr. Carrel also brought us necrotic tissue from two cases of severe gaseous gangrene, from which

<sup>1</sup> Bull, C. G., and Pritchett, I. W., *J. Exp. Med.*, 1917, xxvi, 119.

we isolated the infecting strains. Two strains were isolated from cases of gaseous gangrene occurring in New York City. Five strains were obtained through the kindness of Dr. F. M. Allen from spontaneous gas bacillus infections in diabetic dogs, two others from dog feces, two from human feces, two from market milk, and one from garden soil.

All the strains were obtained in pure culture as indicated by morphological, cultural, and pathogenetic properties, and each strain was tested separately, without animal passage, for artificial toxin production.

#### *Production and Identification of the Toxins.*

Each strain was grown in 0.2 per cent glucose muscle broth for 20 hours at 37°C., and the cultures were centrifuged and filtered.<sup>1</sup> The filtrates were tested for toxicity by intramuscular injection in pigeons. The same quantity of filtrate that was used in the toxicity tests was mixed respectively with *Bacillus welchii* antitoxin and an equal amount of normal serum of the same animal. The mixtures were incubated for 30 minutes at 37°C. and then injected into the breast muscles of pigeons. The degree of toxicity of the filtrate and of the serum-filtrate mixtures was calculated from the degree of the local edema and necrosis which ensued and the quantity of filtrate necessary to cause death.

The procedure described established the fact that all the strains produced toxins which were indistinguishable in pathologic effects from those produced by the five strains described in our first publication, and that an antitoxin made with toxin from one of the former strains completely neutralized the toxins from all the new strains. The toxins of the individual members of the series exhibited, however, a wide range of potency; the lethal dose for pigeons on intramuscular inoculation varied from 0.3 cc. to 3 cc. The lesions and toxic effects were, nevertheless, of the same quality. The toxin-producing power of the less active strains was materially increased by animal passage, and an indication was obtained that a direct relation between infectiosity and toxin production exists. However, the latter point demands a more systematic study than we have yet been able to give it.

To summarize, we may state that twenty-two new strains of *Bacillus welchii* have been secured from widely different sources and studied with regard to morphological, cultural, and pathogenetic properties and toxin production. They have by these means been identified as typical members of the *Bacillus welchii* group of bacilli. Moreover, each strain produced a toxin which was qualitatively indistinguishable in physical and biological properties from the toxins produced by any other members of the series, although the different toxins manifested varying degrees of potency.

Up to the present time we have studied twenty-seven strains of *Bacillus welchii* from widely different sources and have found all to produce a toxin in common.

#### *Factors Influencing the Artificial Production of Bacillus welchii Toxin.*

In our first publication<sup>1</sup> the following method was given for obtaining the toxin *in vitro*: To plain beef infusion broth in 10 cc. quantities in test-tubes are added several fragments of sterile skeletal muscle of the pigeon or rabbit. The tubes, having been proved sterile, are inoculated with *B. welchii*, overlaid with paraffin oil, and enclosed in a vacuum jar from which the oxygen is then exhausted. After an incubation of from 18 to 24 hours, the contents of the culture tubes are centrifuged and the fluid portion of the culture is passed through a Berkefeld N candle. The filtrate contains the toxin. It was stated also that to obtain the most potent product not more than 0.1 per cent glucose should be present, and that the incubation should not exceed 24 hours.

Since then we have been able to carry out a more extensive and systematic investigation of the influence of various factors on artificial toxin production.

Certain technical precautions are necessary in determining the influence of different factors on the production of the toxin *in vitro* in order to eliminate extraneous influences. In the first place, the culture with which the media are inoculated must be controlled with regard to age, the medium in which it is grown, and the number of artificial generations since animal passage. Then the different lots of medium in which toxin is to be produced for the purpose of comparison should be inoculated simultaneously and with the same quantity of culture. Errors may arise in the process of collecting the filtrates. A portion of the toxin is always held back by the filter-

ing candles, and if they are not perfectly clean and open, a large percentage of the toxin may be retained. Under the best conditions different candles seem to retain different amounts of toxin. The following procedure has been followed in this work: A good toxin-producing strain (617 d, Simonds) was used. The strain was of maximum virulence as the result of a number of animal (pigeon) passages. A pigeon was inoculated with an acutely lethal dose of the culture early in the morning of the day preceding the beginning of an experiment. Immediately after the death of the animal, usually late in the afternoon, tubes of plain beef infusion broth were inoculated with small fragments of the infected muscle and incubated at 37° C. over night. The test media were then inoculated with definite quantities of the bouillon cultures, exhausted in a vacuum jar, and incubated in the usual way. At the end of the planned incubation period, the contents of the culture tubes were centrifuged until the supernatant fluid was free of solid matter. The fluid was then syphoned off and filtered. The filters were proved to be open by testing them with sterile distilled water immediately before use and were recleaned as soon as there was evidence of obstruction of the flow of the fluid through them. In this way consistent results were obtained.

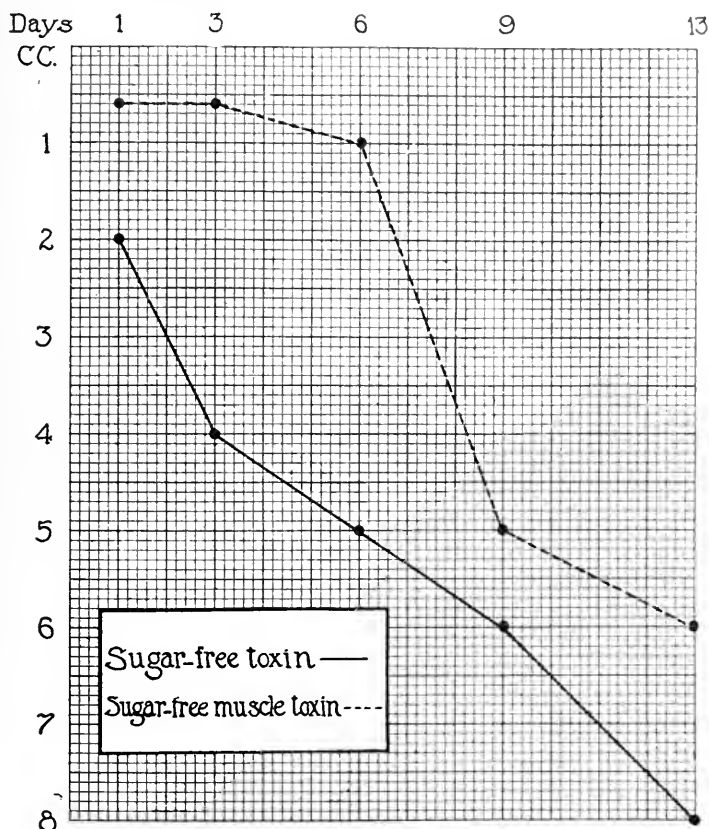
In order to test the potency of the toxin, three different quantities of each toxic product were injected into the breast muscles of three pigeons respectively. The degree of toxicity was estimated from the extent of the edema and necrosis, when sublethal amounts were given, and from the minimal lethal dose. In a number of instances, where the degree of toxicity could not be approximately predicted, the largest quantities given did not cause death or even local lesions. This was especially true of the filtrates from the cultures containing the higher percentages of glucose, in which instances only relative values were obtained.

*Influence of Fresh Muscle and Incubation Time on the Potency of the Toxin.*

We had observed more or less incidentally that the most potent filtrates were obtained after the cultures had been incubated from 18 to 24 hours, and that an extension of the incubation period caused



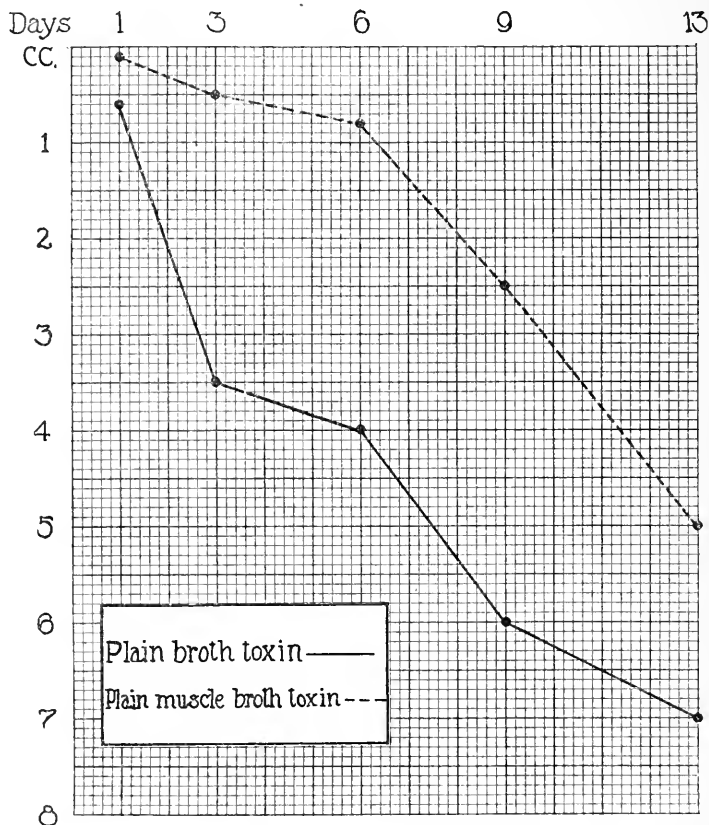
a decrease in toxicity; but the rate and the extent of the decrease in toxicity as the incubation time is prolonged had not been determined. It was, therefore, considered desirable to determine more accurately the relation between incubation time and toxicity. With this end



TEXT-FIG. 1. The media were sugar-free broth and sugar-free broth to which fresh sterile muscle had been added. The tubes were inoculated, freed of oxygen, and incubated in the usual way. The two products are designated as sugar-free toxin and sugar-free muscle toxin respectively.

All the text-figures have the same arrangement. The incubation time of the different specimens of filtrate is represented on the ordinates, while the smallest quantities necessary to produce local lesions and death are represented on the abscissæ. The height of the curves from the base-line represents, therefore, the degree of toxicity.

in view, a variety of media, consisting of various kinds of beef infusion, was inoculated with cultures of *Bacillus welchii* and the filtrates were collected and tested at different intervals, ranging from 16 hours to 13 days.

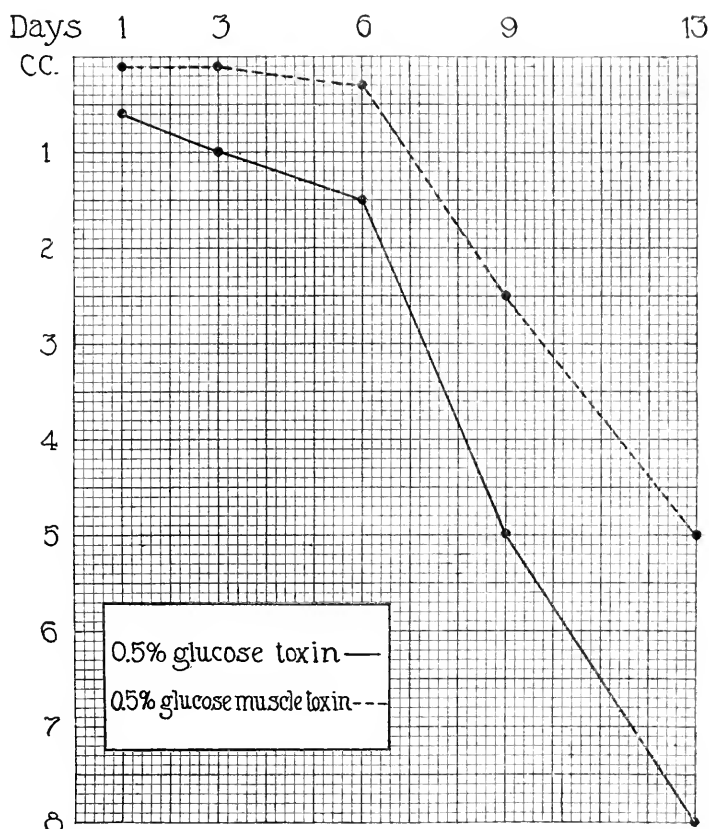


TEXT-FIG. 2. The media were plain broth and plain broth to which sterile muscle had been added. The filtrates are designated as plain broth toxin and as plain muscle broth toxin.

The results obtained may be summarized in the statement that the toxicity of the filtrates is inversely proportional to the incubation time, calculating from the end of the 1st day, and that this general relation obtains independently of the nature of the medium. The

rapidity of the decrease in toxicity, however, is materially influenced by the percentage of glucose in the medium and the presence of raw muscle. The results are presented graphically in Text-figs. 1 to 5.

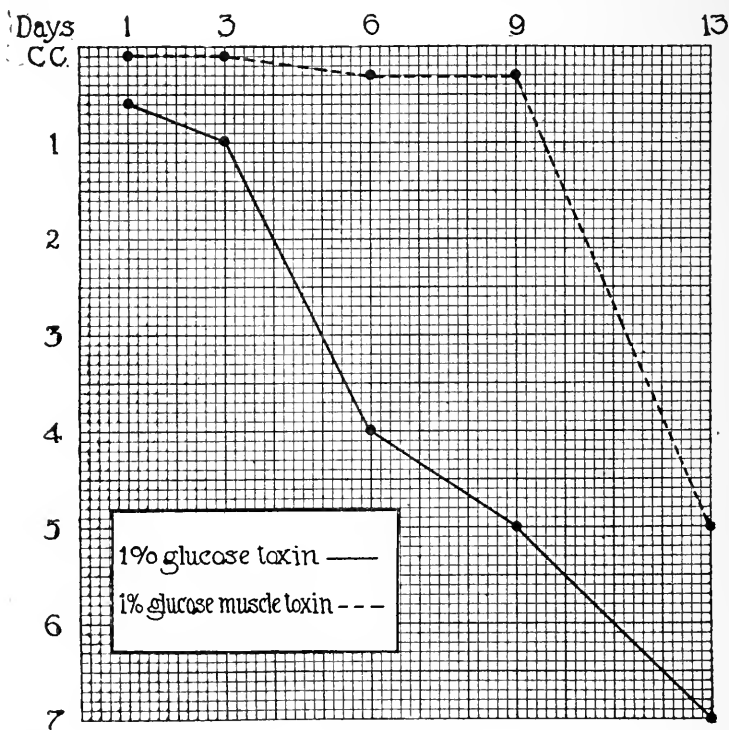
From Text-figs. 1 to 4 it is seen that the toxicity of the filtrates rapidly diminishes as the incubation time is prolonged. The toxicity



TEXT-FIG. 3. The media, 0.5 per cent glucose broth and 0.5 per cent glucose broth plus muscle. The filtrates, 0.5 per cent glucose toxin and 0.5 per cent glucose muscle toxin.

was in every instance highest at the end of the 1st day's incubation. On the 3rd day there was a material decrease in toxicity, especially in the case of the filtrates from the cultures to which muscle had not

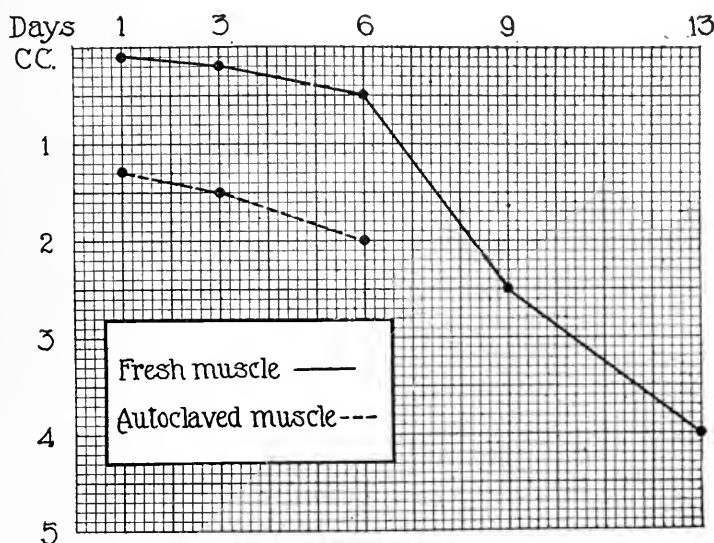
been added. A composite curve of the filtrates from the muscle medium would show that on the 6th day the toxicity had fallen almost 300 per cent. A similar curve of the filtrates from the non-muscle medium would show a decrease in toxicity of 400 per cent. A composite curve including the filtrates from both kinds of media would show a decrease in toxicity of about 1,300 per cent on the 13th



TEXT-FIG. 4. Media, 1 per cent glucose broth and 1 per cent glucose broth plus fresh muscle. Filtrates, 1 per cent glucose toxin and 1 per cent glucose muscle toxin.

day of incubation. It is evident, therefore, that prolonged incubation brings about a destruction of the toxin independently of the nature of the medium, and the most potent toxin is to be obtained at about the end of the 1st day's incubation, the optimum incubation time varying apparently from 18 to 24 hours.

The influence of the muscle upon the potency of the filtrates is very apparent. The 24 hour muscle medium filtrates were in each case about five times as toxic as the corresponding non-muscle filtrates. If the curves had been based on killing power alone, instead of on local effects and killing power, the muscle and non-muscle curves would have been still more widely separated. The toxins formed in the muscle medium seem to have a killing power not possessed by the non-muscle products. Moreover, the non-muscle filtrates lose toxicity more rapidly as the incubation time is prolonged than the



TEXT-FIG. 5. Media, 0.2 per cent glucose broth plus fresh muscle and 0.2 per cent glucose broth plus autoclaved muscle.

muscle filtrates do. The influence of the muscle in the medium is, however, largely a quantitative one. From Text-fig. 5 it is seen that autoclaved muscle will not take the place of fresh muscle.

#### *Glucose, Acidity, and Toxicity.*

It is well known that *Bacillus welchii* produces acids, chiefly butyric, under almost any condition of growth, the quantity produced depending largely upon the amount of fermentable sugars present.

Acid production being so prominent and constant, it is natural that students of this organism should have given special attention to that property. Thus, McCampbell<sup>2</sup> made many experiments on the toxic effects of *Bacillus welchii* cultures and their products and concluded that the acids are of prime importance. This conclusion was based on the observation that his neutralized cultural products were inactive, and that butyric acid in a pure state produced identical results with the acid bacillary products. Stewart and West<sup>3</sup> also state that acid by-products are responsible for all the pathologic effects of *Bacillus welchii* infections. Wright<sup>4</sup> holds similar views and has offered theoretical explanations of the way in which the acids operate in bringing about their destructive effects. In our previous paper<sup>1</sup> we have stated that neutralization with sodium hydroxide did not materially alter the pathologic effects of the cultural products, and there was no direct relation between acidity and toxicity. Further observations have been made on this point, and the results are graphically presented in Text-figs. 6 to 10.

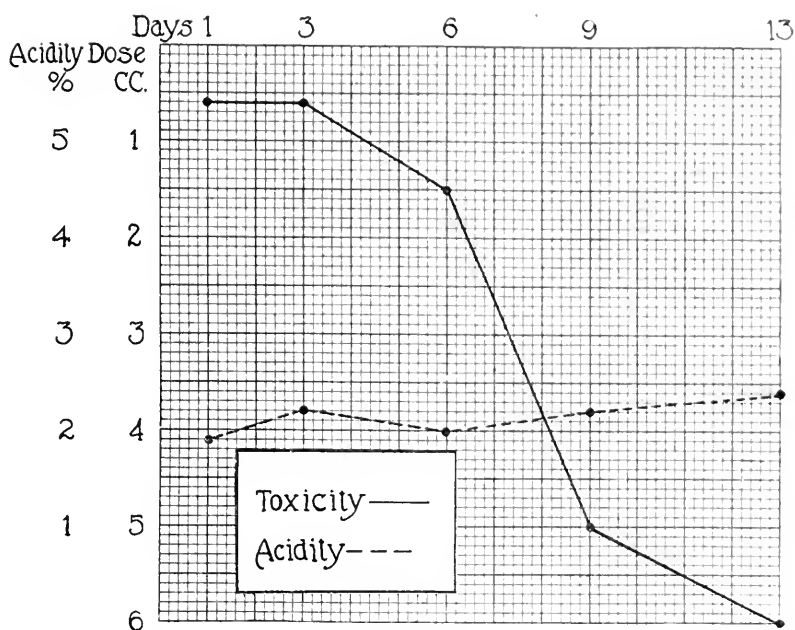
Fragments of fresh rabbit muscle were added to tubes of beef infusion broth containing varying percentages of glucose. A number of tubes of each kind of medium was inoculated with *Bacillus welchii*, a layer of oil added, and the culture tubes were exhausted in a vacuum jar and incubated in the manner already described. Filtrates were collected from a number of the tubes after from 1 to 13 days' incubation and tested for pathologic effects and acidity (Text-figs. 6 to 10).

A study of Text-figs. 6 to 10 discloses the fact that the toxicity of the filtrates is independent of the acidity. The toxic potency of the filtrates rapidly diminishes as the incubation time is prolonged, while the acidity increases or remains constant. In one instance, Text-fig. 9, there was a decrease of 1 per cent in the acidity, but the toxin lost  $\frac{2}{5}$  of its potency. When the acidity is high the filtrates show very slight toxic action. Text-fig. 10 illustrates this point. The acidity was 6.8 per cent on the 1st day, rose to 8 per cent by the 3rd day, and remained constant throughout the experiment. The toxicity curve does not represent the actual potency of the filtrate,

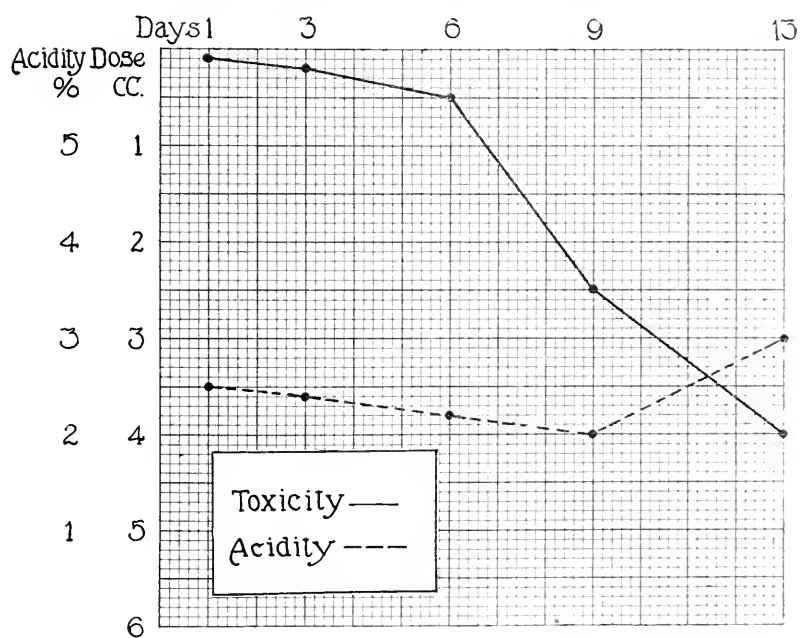
<sup>2</sup> McCampbell, E. F., *J. Infect. Dis.*, 1909, vi, 537.

<sup>3</sup> Stewart, M. W., and West, R., *J. Immunol.*, 1916, i, 189.

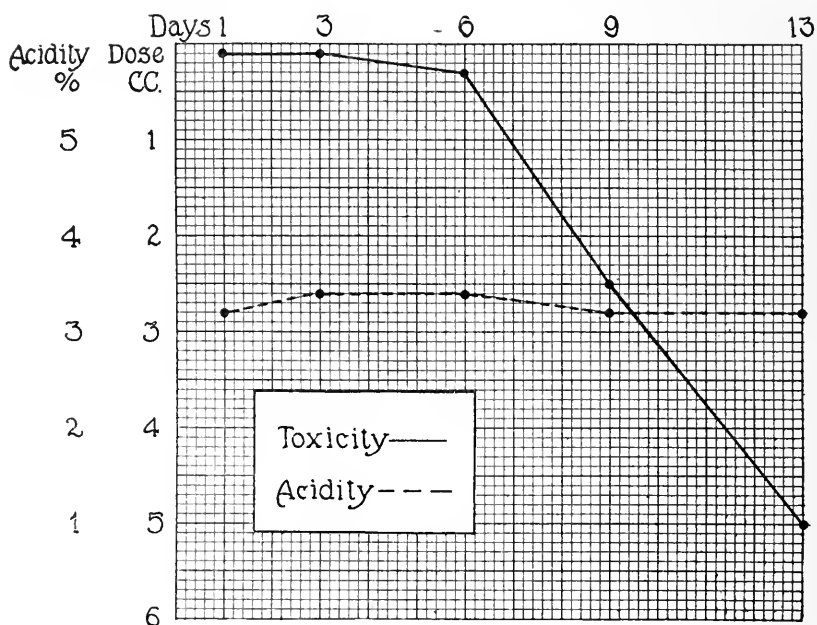
<sup>4</sup> Wright, A. E., *Proc. Roy. Soc. Med.*, 1916-17, x, Occas. Lect., 1.



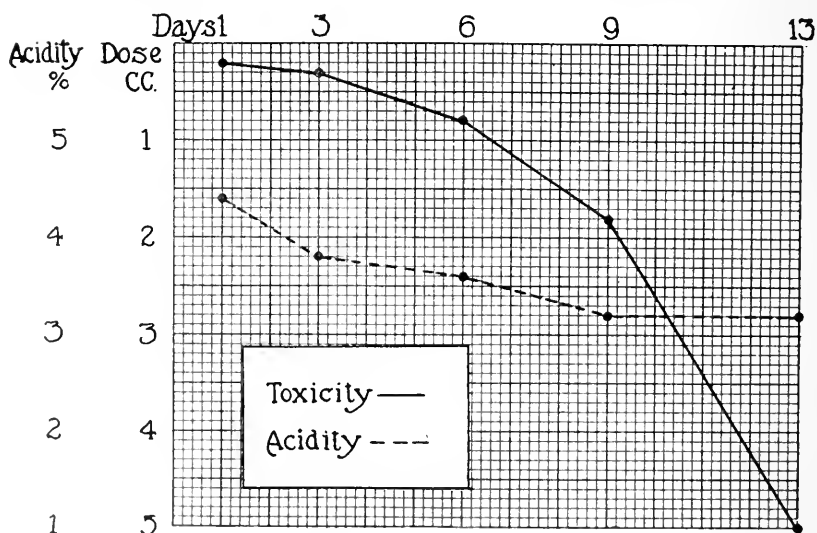
TEXT-FIG. 6. Medium, sugar-free muscle broth.



TEXT-FIG. 7. Medium, 0.2 per cent glucose muscle broth.



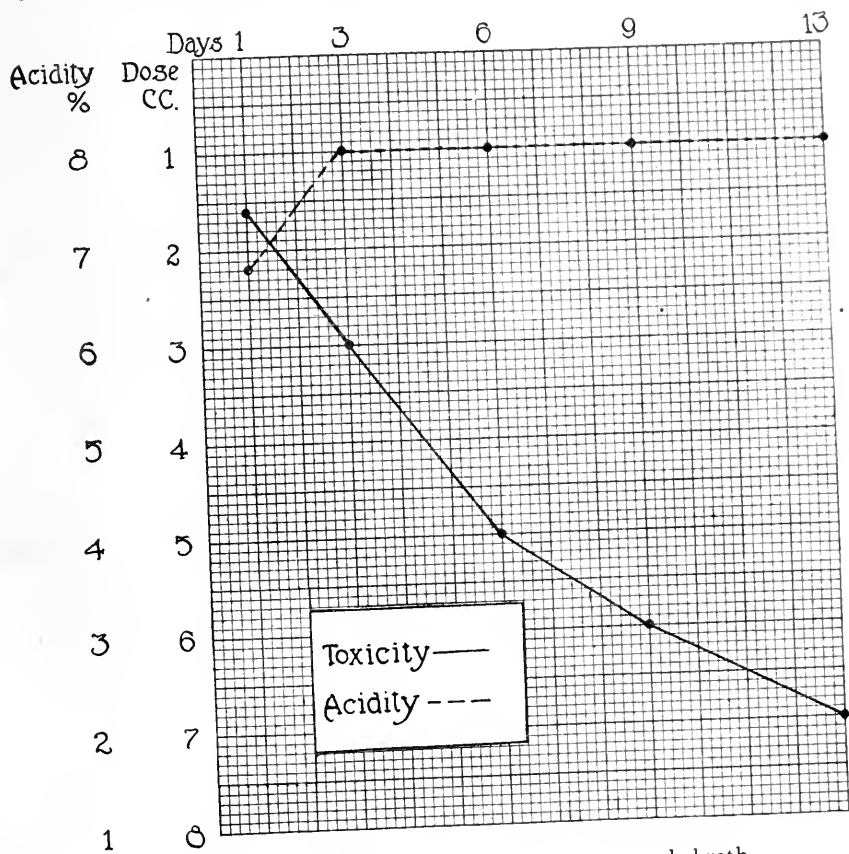
TEXT-FIG. 8. Medium, 0.5 per cent glucose muscle broth.



TEXT-FIG. 9. Medium, 1 per cent glucose muscle broth.



since the quantities used for the animal inoculations were not sufficient to cause death or definite local reactions, the curve being developed from the largest amounts inoculated at the various periods. Beginning with Text-fig. 6, there is a material increase in acidity, which reaches its highest point in Text-fig. 10, while the toxicity increases until Text-fig. 9 is reached, where an abrupt decrease is shown.



TEXT-FIG. 10. Medium, 3 per cent glucose muscle broth.

Text-figs. 6 to 10 may also be used to show the influence of glucose upon toxin production. The glucose content of the medium ranged from 0 to 3 per cent. Since muscle fragments were added to each variety of medium, the percentage of sugar was in each case higher

than is indicated. It is seen that 0.2 per cent glucose causes a material increase in toxicity (Text-figs. 6 and 7). The glucose may be increased to 1 per cent without greatly influencing the potency of the product, while more than 1 per cent has a deleterious effect. The influence of the glucose is, in all probability, non-specific, merely leading to a more luxuriant growth of the bacilli. *As a routine procedure, a 0.2 or 0.3 per cent glucose muscle broth is used for toxin production.*

### *Review of the Literature.*

In our first publication on toxin production by *Bacillus welchii* only brief consideration was given to the work of others on this subject. We propose now to cover the ground somewhat more fully.

In 1904 Kamen<sup>5</sup> reported that in gas bacillus infections in man death occurs under conditions indicating a severe intoxication. He showed also that filtrates from 8 day glucose bouillon cultures of the gas bacillus contained hemolytic substances demonstrable *in vitro*. On the other hand, 10 cc. of the same filtrates caused no lesions on subcutaneous injection in guinea pigs. The author concluded that energetic toxins were not produced in cultures, but that in all probability powerful toxins were produced in the animal body.

Passini's<sup>6</sup> publications on the subject appeared in 1905. He employed a special medium consisting of fresh beef muscle digested with trypsin and sterilized in live steam; to this glucose was added to 1 per cent or more and the air driven out by boiling; or the medium was made by heating the meat-water mixture in an autoclave under from 8 to 9 atmospheres for 1 hour. The medium was then inoculated with the bacilli and incubated for from 14 days to 1 month at 37°C. The filtrates from the cultures were found to be toxic. The toxic effects were manifested immediately on intravenous and intraperitoneal inoculation. When given subcutaneously, edema, necrosis, and sloughing followed. The toxic filtrates resisted heating to 100°C. for 15 minutes, and they were not shown to possess antigenic properties. It is also stated that the filtrates were not toxic if less than 1 per cent glucose was present.

In a general study of the intestinal flora, Metchnikoff gave considerable attention to the gas bacillus.<sup>7</sup> In his case impure cultures were evidently used, since the medium was made with finely chopped beef and tap water, without any attempt at subsequent sterilization. The filtrates from the cultures were found

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Kamen, L., *Centr. Bakteriöl., Its Abt., Orig.*, 1904, xxxv, 554.

<sup>6</sup> Passini, F., *Wien. klin. Woch.*, 1905, xviii, 921.

<sup>7</sup> Metchnikoff, E., *Ann. Inst. Pasteur*, 1908, xxii, 951.

to be toxic, the toxicity being greatest after from 2 to 5 days' incubation. A longer incubation period caused a progressive diminution in toxicity. Heating to 100°C. did not reduce the potency of the toxin obtained; moreover, it was not tested for antigenic properties.

Schultze<sup>8</sup> showed that the disappearance of the nuclei in the organs of animals dying of gas bacillus infection was not due to gas formation, and he inferred that the phenomenon was caused by chemical toxic substances formed by the bacilli.

Korentchevsky,<sup>9</sup> working in Metchnikoff's laboratory, found that filtrates from bouillon cultures of the gas bacillus were toxic for rabbits, especially young rabbits. Symptoms of intoxication appeared in from 1 to 3 hours after intravenous and intraperitoneal injections. The chief symptoms were dyspnea, convulsive movements of the head, opisthotonos, clonic convulsions of the extremities, and paralysis. The effects of intramuscular and subcutaneous injections were not determined. Rabbits and dogs were given large quantities (20 cc.) of the filtrates *per rectum* every 2nd or 3rd day for 2½ weeks. The growth of the animals was arrested, and some lost weight. Agglutinins, precipitins, and fixatins were found in the sera of the rabbits. No mention is made of the thermostability, acidity, or antitoxin-producing properties of the filtrates.

Klose<sup>10</sup> studied 135 cases of gas phlegmon, in 39 of which the Fränkel gas bacillus (*B. welchii*) was present. The chief symptom in the cases was intoxication. Toxic substances were demonstrated in the blood sera of 5 cases of gas phlegmon in man. The clinical observations apparently led to the experimental work.

The organism used in the animal experiments was isolated from a foudroyant case of gas phlegmon in man. Toxic substances were demonstrated in the subcutaneous exudates occurring in infected guinea pigs and in Berkefeld filtrates from 5 per cent glucose broth cultures at the end of 14 days' incubation. Subcutaneous injections in guinea pigs gave rise to edema, discoloration, falling of

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<sup>8</sup> Schultze, W. H., *Virchow's Arch. path. Anat.*, 1908, xciii, 419.

<sup>9</sup> Korentchevsky, W., *Ann. Inst. Pasteur*, 1909, xxiii, 91.

<sup>10</sup> After our first publication on *B. welchii* toxin and antitoxin appeared, our attention was called to an abstract in the *Chemical Abstracts*, July 20, 1917, of an article by F. Klose entitled: "Ueber Toxin- und Antitoxinversuche mit dem Fränkelschen Gasbrandbacillus." This abstract was made by Julian H. Lewis from an abstract which appeared in the *Chem. Zentr.*, 1916, ii, 24. The original article appeared in the *Munch. med. Woch.*, 1916, lxiii, 723. This journal could not be found in any of the libraries in New York City or elsewhere in the United States. The abstracts contained only a few summary statements without any experimental details, and no idea of the nature of the work could be formed. The original article has, however, recently come to our hands through the kindness of Dr. J. E. J. King, who returned from Germany in April after rupture of diplomatic relations between the United States and Germany. Because of this accidental circumstance, it is possible to consider the work here.

hair, necrosis, and sloughing; intraperitoneal and intravenous injections caused immediate symptoms, tremors, failure of respiration, and death. The toxic substances were quite thermostable, since 80°C. for 1-hour did not reduce their toxic effects. A horse was immunized as follows: 2,415 cc. of filtrate from 5 day 5 per cent glucose broth cultures were given intravenously during 17½ weeks and after a rest period of 6 weeks, 1,780 cc. of filtrate were given during 6½ weeks. The immune serum protected guinea pigs against three lethal doses of bacilli; 2 cc. of serum were injected at the site of infection 24 hours previously. Infections were controlled by giving the immune serum 2 hours after the guinea pig had been infected.

We have made repeated attempts to produce *Bacillus welchii* toxin artificially according to Klose's method. The filtrates were collected both after 5 days' and 14 days' incubation. Large quantities (5 cc.) of the filtrates failed to cause death or necrosis on intramuscular injection in guinea pigs and pigeons. There was, in some instances, a slight tumefaction of the tissues at the point of inoculation. In the light of these results it is somewhat difficult to explain the pathologic effects of Klose's filtrates. The neutralization and protection experiments, on the other hand, can be readily explained by the fact, observed by us, that a certain number of horses possess natural *Bacillus welchii* antitoxin. Klose did not determine the natural antitoxin content of his horse's serum.

The work of Weinberg and Séguin<sup>11</sup> was referred to in our first publication. These authors made numerous reports on the production of specific antitoxic sera for the bacillus of malignant edema (*vibrio septique*) and for *B. œdematiens* (Weinberg). They reported also that a potent specific antibacterial serum had been produced by immunizing horses with living cultures of *B. welchii* (*B. perfringens*). Frequent mention is made of a *B. perfringens* toxin, but nothing is stated concerning its preparation, or physical or biological properties, but a definite statement is made that it has not been found possible to prepare an antitoxic serum for the organism. Finally, these authors have recently reported on the clinical application of their antimicrobial serum<sup>12</sup> for *B. perfringens* (*B. welchii*) infection.

#### SUMMARY.

Twenty-two additional strains of *Bacillus welchii* have been collected from widely different sources and tested with regard to toxin

<sup>11</sup> Weinberg, M., *Proc. Roy. Soc. Med.*, 1916, ix, Occas. Lect., 119.

<sup>12</sup> Weinberg, M., and Séguin, P., *Compt. rend. Acad.*, 1917, clxv, 199.

production. Each strain produces a toxin which, on animal inoculation, gives rise to lesions comparable in every respect to those produced by the toxins previously reported on,<sup>1</sup> and each toxin was neutralized by an immune (antitoxic) serum produced with one of the former toxins. The toxins obtained from the several individual strains varied in potency, the lethal dose ranging from 0.3 to 3 cc.

Experiments have been made to determine the influence of fresh muscle and glucose on toxin production and the relation of acidity to toxicity in the filtrates. It has been found that the addition of fresh muscle to the medium increases the potency of the toxin five-fold. Autoclaved muscle is without effect. Beef infusion broth containing 0.2 to 1 per cent glucose gives a more potent product than sugar-free broth, while when higher percentages are employed the toxin production is lowered. There is no direct relation between acidity and toxicity, the most acid products manifesting little or no toxic action. In every medium used for culture the potency of the filtrates rapidly diminished after 24 hours' incubation, while the acidity increased or remained constant. The exception to this rule has been pointed out.

The most active toxin is obtained by growing a virulent strain of the bacilli in a 0.2 or 0.3 per cent glucose broth to which fragments of fresh muscle have been added, and collecting the filtrate after from 18 to 24 hours' incubation.

A review of the literature on the pathogenic effects and toxic products of *Bacillus welchii* and on the results of immunization of animals with the bacilli or toxic products does not indicate that the exotoxic nature of *Bacillus welchii* had been previously determined or an antitoxic serum in the true sense produced.

The antitoxin for *Bacillus welchii* toxin can apparently be prepared from a single strain of the organism which yields under the conditions described a high titer of toxin, and this antitoxin can be employed to combat infection with or prevent infection by any strain whatever of the bacillus.



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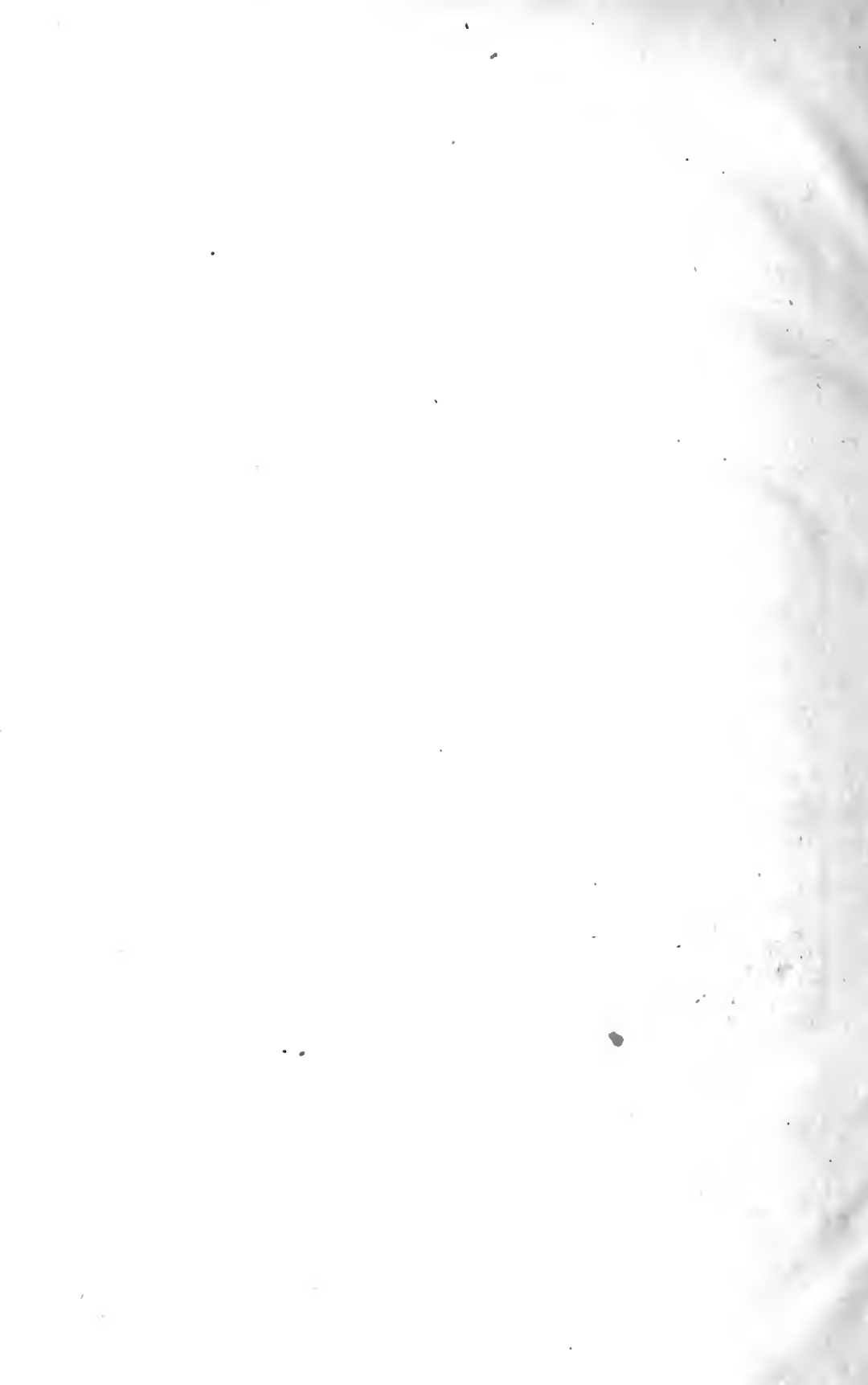
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